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TABLE OF CONTENTS

ENDOCRINOLOGY

The Effect of Adrenergic and Ganglionic Blockers upon the L-Dopa-Stimulated Release of Glucagon in the Rat	D. T. George, P. T. Bailey	1
Stimulation by Gastric Inhibitory Polypeptide of Insulin and Glucagon Secretion by Rat Islet Cultures	W. Y. Fujimoto, J. W. Ensinck, F. W. Merchant, R. H. Williams, P. H. Smith, D. G. Johnson	89
In Vitro Effects of Melatonin and Serotonin on Adrenal Steroidogenesis	T. F. Ogle, J. I. Kitay	103
Antenatal Diagnosis of Cretin Lambs by Measurement of Amniotic Fluid Thyrotropin	D. R. Hollingsworth, S. L. Davis, I. J. Chopra, R. P. Belin, M. C. Reid	106
Effects of Thyrotropin-Releasing Hormone on Plasma Thyroxine in the Calf: Comparison of Intraventricular and Intravenous Injection Routes	L. Hedlund, M. M. Lischko, A. J. Tollerton, S. G. Doelger, H. D. Johnson, D. L. Eklund	134
Differential Effects of Stress on Release of Thyroid-Stimulating Hormone in Young and Old Male Rats	J. W. Simpkins, C. A. Hudson, J. Meites	144

ENZYMOLOGY

Spectrophotometric Assay of Catalase with Perborate as Substrate	J. F. Thomson, S. L. Nance, S. L. Tollaksen	33

IMMUNOLOGY

The Radiolabeling of Lymphocytes and Tumor Cells with ^{111}Indium	P. Frost, J. Smith, H. Frost	61
Effects of Rheumatoid Factor and Complement on Soluble Immunoglobulin G Complexes: $^{51}CrCl_3$ Labeling and Precipitation Studies	R. Turner, H. Mashburn, J. Herron, R. Collins, J. Kaufmann	75
Clinical and Serological Evaluation of a Meningococcal Polysaccharide Vaccine Groups A, C, and Y	J. D. Farquhar, W. A. Hankins, A. N. DeSanctis, J. L. DeMeio, D. P. Metzgar	79
Inhibition of Eosinophilotaxis by Chloroquine and Corticosteroids	C. A. Gauderer, G. J. Gleich	129
Vaccination and Revaccination with Polyvalent Pneumococcal Polysaccharide Vaccines in Adults and Infants	J. M. Borgoño, A. A. McLean, P. P. Vella, A. F. Woodhour, I. Canepa, W. L. Davidson, M. R. Hilleman	148
Effect of Penicillamine on the Proliferative Response of Human Lymphocytes	P. Merryman, I. A. Jaffe	155

MICROBIOLOGY

Inhibition of Interferon Induction in Mice by Mycoplasmas and Mycoplasmal Fractions	B. C. Cole, P. S. Lombardi, J. C. Overall, Jr., L. A. Glasgow	83
Effect of Freezing of Human Fecal Specimens upon the Isolation of Nuclear Dehydrogenating Clostridia	A. R. Hedges, K. Hedges, B. S. Reddy	94

NUTRITION

Studies on the Metabolism of Glycerol by Hyperlipemic and Normolipemic Rats	C. D. Berdanier, R. B. Tobin, V. DeVore, R. Wurdeman	5

Erythrocyte Glucose-6-Phosphate Dehydrogenase PD) Deficiency on Light-Induced Riboflavin Defi- y in the Neonate — R. LOPEZ, D. S. GROMISCH, H. S. DOLE, J. M. COOPERMAN 41

nduced Hyperuricemia and Orotic Aciduria in Mice — S. C. MANGOFF, J. A. MILNER 110

nino Acid Activity of D- and L-Homocysteine for :s — J. M. HARTER, D. H. BAKER 139

PATHOLOGICAL PHYSIOLOGY

of Cystic Fibrotic and Heterozygous Serum on Rat um — J. P. GILMORE, M. DAVIS, G. E. GIBBS 70

n of Renal Hypertension in Adrenalectomized Dogs onstant Hormone Replacement Therapy — B. E. WATKINS, J. O. DAVIS, R. H. FREEMAN, G. A. STEPHENS 116

PHARMACOLOGY

of Angiotensin-Converting Enzyme by SQ 14,225 esthetized Dogs: Hemodynamic and Renal Vascular ts — V. S. MURTHY, T. L. WALDRON, M. E. GOLDBERG 121

PHYSIOLOGY

Plasma Concentration and Urinary Excretion of Na in the Rat — S. SOLOMON, H. H. BENGELE, W. D. SMITH 12

on of a β-Adrenergic Response in Rats Treated nically with Ethynyl Estradiol — M. J. FREGLY, T. N. THRASHER, S. A. MACARTHUR, D. L. KELLEHER 18

o Study of Pancreatic Ductal Cells — M. SINGH, M. M. PARKS, P. D. WEBSTER, III 23

in the Canine Lung which Respond to Vascular ure Changes — F. O. IGLER, R. L. COON, E. J. ZUPERKU, J. P. KAMPINE 36

nce of Bile Acid and Ouabain Hepatic Uptake: es in the Newborn Rat — C. D. KLAASSEN 66

of Variables Influencing Urinary pCO_2 during CO_3 and Water Loading in Normal Man — G. O. PEREZ, J. R. OSTER, R. E. SONNEBORN, J. CASSADY, C. A. VAAMONDE 97

RADIOBIOLOGY

itivity of the Human Parotid Gland — I. L. SHANNON, J. N. TRODAHL, E. N. STARCKE 50

n of Mercury from Ligated Segments of the Rat ointestinal Tract — L. B. SASSER, G. E. JARBOE, B. K. WALTER, B. J. KELMAN 57

REPRODUCTION

Calcium on Motility and Fertilization by Rat Sper- oa *in Vitro* — B. K. DAVIS 54

TOXICOLOGY

of the Fluoride-Induced Urinary Concentrating De- Rats — G. M. WHITFORD, G. I. STRINGER 44

VIROLOGY

Resistance of C3H/HeJ Mice to Lethal Challenge with Herpes Simplex Virus — H. KIRCHNER, H. M. HIRT, D. L. ROSENSTREICH, S. E. MERGENHAGEN ... 1

Effect of Sindbis Virus Infection on Hydrocortisone-Induced Hepatic Enzymes in Mice — R. N. MOORE, L. J. BERRY, R. F. GARRY, M. R. F. WAITE 1

THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 1–4 (1978)

fect of Adrenergic and Ganglionic Blockers upon the L-Dopa-Stimulated Release of Glucagon in the Rat[1] (39978)

DAVID T. GEORGE AND PAUL T. BAILEY

States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

:retory function of the endocrine s altered by a number of neutro- rs including epinephrine (1), hrine (2), acetylcholine (3), ser-), dopamine (5), and L-dopa (6, neural control of the endocrine has been reviewed in detail (8); ear to be at least two mecha- action associated with these neu- ters. The most extensively stud- anism involves the stimulation sion of α- and β-adrenergic recep- nses (8). Another mechanism function, independent of the ad- ervous system, via the choliner- dopaminergic (5) pathways. In it data from our laboratory have at L-dopa elicits the release of and promotes hyperglycemia in 6) and man (7).

ie mechanism responsible for the luced release of glucagon is un- tudies were designed to define ipletely the role of the sympa- rvous system in this phenome- ntolamine and propranolol were block the α- and β-adrenergic respectively. In addition, dopa- given to determine if it would : L-dopa-induced release of glu-

ium tartrate, a ganglionic vas given alone and in conjunc- L-dopa to ascertain the role of omic ganglia in mediating the L- ced release of glucagon.

Materials and methods. One hundred and twenty 14-hr-fasted, 180- to 220-g male Fisher–Dunning rats were randomly assigned to 12 groups of 10 animals each. Table I presents the experimental design used to assess the influence of L-dopa as well as selected adrenergic and ganglionic blockers upon the L-dopa-induced release of glucagon. The first study consisted of 30 rats which had no pretreatment, whereas in studies 2, 3, and 4 each rat received a prior intraperitoneal (ip) injection of phentolamine, propranolol, pentolinium tartrate 20 min before the intravenous (iv) injection of L-dopa or saline. Following sodium pentobarbitol (50 mg/kg, ip) anesthesia a midline laparotomy was performed exposing the hepatic portal and left femoral veins. A 21-gauge pediatric iv injection set was inserted into the hepatic portal vein, the basal (time = 0) 1.5-ml blood sample was taken, and the catheter was flushed with 1.5-ml of saline. Immediately after obtaining the basal sample a 25-gauge needle was inserted into the femoral vein for the administration of L-dopa, dopamine, or saline over a 30-sec time period. Two subsequent blood samples were obtained from the portal vein of each rat 5 and 15 min after the iv injection. Samples were immediately transferred to iced, capped, polypropylene test tubes containing 15 mg of disodium ethylenediaminetetraacetic acid and 1000 U (kallikrein inactivator units) of Trasylol/ml of whole blood. The samples were centrifuged at 4° for 15 min at 1000g; the plasma was separated and stored at −20° until assayed for glucose (9), glucagon (10), and insulin (11). Data were analyzed using Student's t test for paired or unpaired variates; $P < 0.02$ was the criterion of significance.

Results. The first study evaluated the effects of L-dopa, dopamine, and saline

ducting the research described in this investigators adhered to the "Guide for id Use of Laboratory Animals," as pro- / the Committee on the Revision of the .aboratory Animal Facilities and Care of : of Laboratory Animal Resources, Na- :arch Council. The facilities are fully ıy the American Association for Accred- boratory Animal Care.

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upon the portal vein concentrations of glucagon, insulin, and plasma glucose in the rat. The iv administration of 10 mg/kg of L-dopa or dopamine induced a significant ($P < 0.01$) increase in portal plasma glucagon at 5 and 15 min which was accompanied by a significant ($P < 0.001$) elevation in portal plasma glucose (Fig. 1). The saline data demonstrate that the anesthesia and animal manipulation had no effect upon the subsequent portal vein concentrations of glucagon and glucose. Portal vein concentrations of insulin determined during the height of the glucagon response at 5 min were unaltered by L-dopa and dopamine treatments when compared to saline controls (Table I).

The second study evaluated the effect of an α-adrenergic receptor blocking agent on the L-dopa-induced release of glucagon in the rat. As shown in Table II, phentolamine blocked the L-dopa-stimulated

TABLE I. EXPERIMENTAL DESIGN, DRUG TREATMENTS, AND THE PORTAL PLASMA VEIN INSULIN[a] CONCENTRATIONS 5 min AFTER INJECTION OF L-DOPA OR DOPAMINE.

Study No.	Treatments (dose)	Portal plasma insulin (μU/ml)	P compared to controls[b]
1	Saline controls[c]	20 ± 2	
	L-Dopa (10 mg/kg, iv)	31 ± 7	NS
	Dopamine (10 mg/kg, iv)	16 ± 5	NS
2	Phentolamine controls (4 mg/rat, ip)	243 ± 82	<0.01
	Phentolamine + L-dopa	248 ± 27	<0.001
	L-Dopa controls	48 ± 10	
3	Propranolol controls (1 mg/rat, ip)	58 ± 13	NS
	Propranolol + L-dopa	33 ± 5	NS
	L-Dopa controls	28 ± 4	
4	Phentolinium tartrate controls (5 mg/rat, ip)	53 ± 12	NS
	Phentolinium tartrate + L-dopa	36 ± 6	NS
	L-Dopa controls	31 ± 6	

[a] Ten rats per group; mean ± SEM.
[b] Saline, study 1; L-dopa, studies 2-4.
[c] Saline was given iv in a volume equivalent to L-dopa.

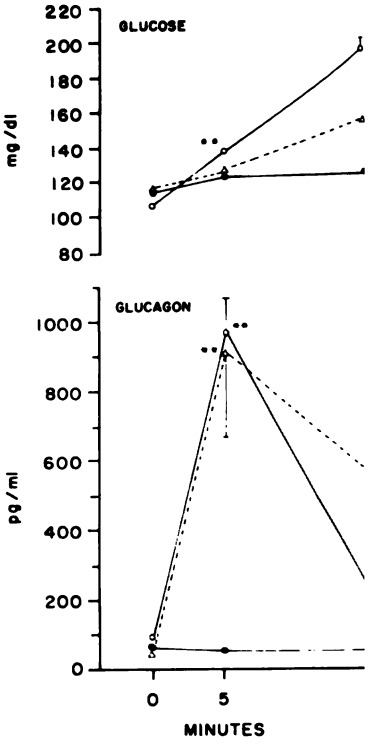

FIG. 1. Effect of dopamine (△) and L-do plasma glucose and glucagon levels in ra 0.01 and $^{**}P < 0.001$ by paired t test co time 0 values for each treatment. Stand are plotted when $P < 0.01$ by unpaired compared to saline controls (●).

release of glucagon and glucose. ment with phentolamine elicited cant ($P < 0.001$) elevation of port insulin (Table I). L-dopa had n upon the phentolamine-induced in sponse.

The third study evaluated the of prior β-adrenergic receptor blo tablished with propranolol (1 there was no effect upon the port glucagon. However, it did sig elevate plasma glucose at 5 (P and 15 ($P < 0.001$) min when con basal levels (Table II). During β blockade, L-dopa was still an stimulator of glucagon release at min ($P < 0.01$) but there was a s muting of the response ($P < 0.($ compared to the L-dopa controls (Although propranolol induced a tion of plasma glucose, the L-dop

ABLE II. PORTAL PLASMA LEVELS OF GLUCAGON AND GLUCOSE IN RATS DURING ADRENERGIC AND GANGLIONIC BLOCKADE AT 0, 5, AND 15 min AFTER L-DOPA.

Group	0 Minutes		5 Minutes		15 Minutes	
	Glucagon (pg/ml)	Glucose (mg/dl)	Glucagon (pg/ml)	Glucose (mg/dl)	Glucagon (pg/ml)	Glucose (mg/dl)
amine + saline	73 ± 7	114 ± 2	76 ± 6[a,c]	118 ± 3	93 ± 12[b]	120 ± 6[b]
amine + L-dopa	66 ± 5	98 ± 6	82 ± 10[c]	130 ± 9	90 ± 10[b]	122 ± 7[a]
controls	118 ± 14	107 ± 3	1169 ± 163[f]	140 ± 3[f]	255 ± 33[e]	187 ± 6[f]
ıolol + saline	114 ± 21	122 ± 5	73 ± 5[c]	144 ± 4	100 ± 16	161 ± 3[b]
ıolol + L-dopa	68 ± 5	120 ± 4	451 ± 80[b]	150 ± 6	209 ± 68[a]	188 ± 9
controls	76 ± 11	121 ± 10	773 ± 88[f]	138 ± 14	155 ± 27[d]	208 ± 17[f]
ıium tartrate +	75 ± 11	102 ± 4	46 ± 2[c]	111 ± 3[b]	51 ± 5[b]	120 ± 3[b]
ıium tartrate + L-	46 ± 3	111 ± 9	688 ± 99	118 ± 5[b]	148 ± 20[c]	176 ± 10[b]
controls	92 ± 12	114 ± 5	807 ± 103[f]	151 ± 11[e]	246 ± 43[f]	213 ± 8[f]

tistically significant, as follows: (a) $P < 0.02$ vs L-dopa controls; (b) $P < 0.01$ vs L-dopa controls; (c) P l vs L-dopa controls; (d) $P < 0.025$ vs 0-time values; (e) $P < 0.01$ vs 0-time values; (f) $P < 0.001$ vs 0- ues.

significantly ($P < 0.01$) higher than opranolol controls (Table II). Portal ı insulin levels tended to be higher ere not significantly altered by pre- ent with propranolol (Table I).

ganglionic blocker pentolinium tar- was examined in the fourth study. It arent in Table II that this agent had ect upon the portal plasma concen- ıs of glucagon or the L-dopa-me- glucagon response. Ganglionic de diminished the plasma glucose ıse to L-dopa at 5 and 15 min. Portal of insulin in pentolinium tartrate- l rats were not significantly differ- ım the L-dopa controls (Table I).

ussion. The data presented demon- that L-dopa induces a significant : of glucagon which is detectable at concentrations in portal plasma 5 min. The magnitude and timing s response in rats is in agreement previous studies in monkeys (6). dopamine is unable to cross the brain barrier (13) but mimics the L- nduced release of glucagon, the ef- f these compounds appears to be eral, and not central nervous sys- ediated as postulated previously (6, ıe inability of ganglionic blockade to the L-dopa-induced glucagon release es that the response probably is not ed via the autonomic ganglia. Since been shown that L-dopa may be olized within the pancreas to norep- inephrine and epinephrine (14) and that both the α- and β-adrenergic blockers are effective suppressors of the glucagon release, it appears likely that the response is mediated via the adrenergic receptors. These findings are consistent with the report of Harvey *et al.* (15), but in direct contrast to our previous reports in monkeys. The reasons for these differences appear to be, first, species differences, or second, a more likely explanation, differences in dosage of the α- and β-blockers. Although our previous studies in monkeys indicated effective blockade of some parameters, it was not sufficient to alter the insulin release prior to L-dopa treatment (6). In the present study the α-blocker engendered a striking increase in plasma insulin. Furthermore, Wilson *et al.* (5) have reported inhibition of the L-dopa-mediated insulin responses with similar high doses of α-blockers.

An alternate explanation for the L-dopa-mediated endocrine pancreas responses, as proposed by others (5, 7), is the presence of dopaminergic receptors within the endocrine pancreas. However, recently Lorenzi *et al.* (16) have reported that this is not a likely explanation since a known dopaminergic agonist, apomorphine, was unable to stimulate the release of glucagon and insulin in man.

Therefore it appears likely that the response is due to metabolism of L-dopa and dopamine to norepinephrine and epineph-

rine and is a result of stimulation of the α- and/or β-adrenergic receptors.

Summary. To help characterize the L-dopa-mediated release of glucagon, rats were given either L-dopa or dopamine (10 mg/kg) intravenously; portal plasma levels of insulin, glucagon, and glucose were measured in the presence and absence of adrenergic and ganglionic blockers.

The α-adrenergic blocker (phentolamine) suppressed the glucagon and glucose responses to L-dopa and increased plasma insulin levels. β-Adrenergic blockade with propranolol ameliorated the L-dopa-mediated glucagon and glucose responses but had no effect on plasma insulin levels.

Ganglionic blockade with pentolinium tartrate was ineffective and did not alter any of the L-dopa-mediated responses of glucagon, insulin, or glucose.

These data indicate that L-dopa and dopamine probably act via the sympathetic nervous system through known pathways enhancing the release of glucagon. Furthermore they substantiate the fact that the adrenergic nervous system is involved in the regulation of the endocrine pancreas.

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lies on the Metabolism of Glycerol by Hyperlipemic and Normolipemic Rats[1] (39979)

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viously, it has been reported that yperlipemic, maturity-onset diabetic rat (1) had significantly higher levels um free glycerol than the normoli-, nondiabetic Wistar rat (2). The of this glycerol was not elucidated as a causal relationship between the ed glycerol and hepatic lipogenesis .

ently, Lin *et al.* (3) reported that g large quantities of glycerol had effect on hepatic lipogenesis as mea- by the incorporation of tritium into acids, while inducing an increase in ctivities of citrate cleavage enzyme .1.3.8), malic enzyme (EC 1.1.1.40), atty acid synthetase. If one assumes his high dietary glycerol intake re- in elevated serum glycerol levels, ould also assume that this experi- would produce a rat which might be r to the BHE rat at least in so far as elationship between serum glycerol and hepatic enzyme activity was ned. One might conclude, therefore, he elevations in serum glycerol re- l to occur in the BHE rat do not a causal relationship to the previ- reported enhanced lipogenesis in the rat. This hypothesis, of course, raises uestion of the significance of elevated glycerol levels. It may well be that abnormality" is merely characteristic more fundamental error in metabo- which ultimately results in the devel- nt of hyperlipemia, maturity-onset es, and vascular disease, diseases ent in the 300- to 400-day-old BHE rat (1, 4). For this reason we decided to investigate glycerol metabolism in the BHE rat compared to the normal Wistar rat. We restricted our inquiries to the metabolism of the liver since previous studies have shown that the major differences in lipid metabolism (as well as glucose metabolism) were observed in this organ. The study consisted of a series of experiments designed to answer the following questions in sequence: (i) Using a homogeneous BHE population, could the previously reported elevation in serum free glycerol be demonstrated? (ii) Were these differences in serum free glycerol due to (a) strain differences in heparin-induced lipoprotein lipase activity or (b) differences in the capacity of the liver to metabolize varying levels of incoming glycerol? (iii) Were there differences in the hepatic metabolism of glycerol? Using BHE and Wistar rats we found that while glycerol can enter the liver cell, its movement from the cytosol to the mitochondria is restricted, necessitating the development of alternate pathways for glycerol metabolism which, in turn, may contribute to, rather than inhibit, the lipogenic capacity of the BHE liver.

Materials and methods. Four experiments were conducted. Each experiment utilized age-matched young male BHE and Wistar[3] rats. The BHE rats used were third- and fourth-generation animals inbred[4] (brother-sister matings) to produce a homogeneous population of matu-

)ported by the BLY Memorial Research Fund Veterans Administration Hospital.

:sent address: Department of Foods and Nu- University of Georgia, Athens, Georgia Dawson Hall).

[3] Wistar rats were purchased from Charles River Breeding Laboratories, Wilmington, Massachusetts.

[4] The original BHE animals were purchased from Flow Laboratories, Dublin, Virginia. The BHE colony has been moved from this facility to the NIH Animal Resource Center.

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rity-onset diabetic, hyperlipemic rats. These rats differed from the rats used in previous work (1, 4) in that they were more uniform in response to carbohydrate feeding and in the development of the strain characteristics. The rats were housed in stainless-steel cages in a temperature-humidity-controlled room having equal 12-hr periods of light and dark. Food[5] and water were always available.

In the first experiment, two groups of 10 BHE and Wistar 50-day-old rats were anesthesized with sodium barbital (60 mg/kg) and blood was withdrawn by heart puncture. The serum was collected after centrifugation in the cold and used for the determination of lactate (5), pyruvate (6), β-hydroxybutyrate (7), acetoacetate (7), and glycerol (8). The second experiment utilized BHE and Wistar rats approximately 75 days old. Four groups of rats, six per group, from each strain were anesthesized with sodium barbital. Heparin (2000 units) was injected via the femoral vein, the inferior vena cava was catheterized via the jugular, and the portal vein was exposed. Saline or glycerol (35, 70, or 140 μmole/100 g body weight) was injected at 0 time into the portal vein. At 5 min prior to the injection and at 1, 3, 5, 10, 20, and 30 min after injection, blood was withdrawn from the catheter inserted into the inferior vena cava. The serum from this blood was collected after centrifugation and used for the determination of glycerol (8). Immediately after the last blood sample was drawn, the liver was quickly excised and clamped between copper plates precooled in liquid nitrogen. Perchlorate extracts of the frozen liver powders were prepared (9) and used for the determination of glycerol and α-glycerophosphate (10). Total lipid content of these powders was also determined (11).

In the third experiment, two groups of 50-day-old BHE and Wistar rats were killed by cervical dislocation, and the livers were excised and clamped between two copper plates precooled in liquid nitrogen. Less than 7 sec elapsed between the start of the cervical dislocation and the clamping of the tissue. Liver powders were prepared as described above and used for the determination of glycerol, α-glycerophosphate, dihydroxyacetone phosphate (9), 3-phosphoglycerate (9), and adenine nucleotides (9). Phosphorylation states were calculated according to the equations of Veech *et al.* (12).

In the fourth experiment, two groups of 50-day-old BHE and Wistar rats (nine rats per group) were killed by decapitation and the livers excised. Mitochondria were prepared according to the procedures of Johnson and Lardy (13) and used for the determination of mitochondrial α-glycerophosphate dehydrogenase (EC 1.1.99.5) (14) and for the activity of the α-glycerophosphate shuttle.[6]

Results of the different experiments were summarized and strain differences determined using Student's *t* test. Differences with a probability of less than 0.05 were considered significant.

Results. No significant differences in serum lactate and pyruvate were observed (Table I). There was, however, a significant increase in β-hydroxybutyrate levels and total ketones in BHE rats compared to Wistar rats. Acetoacetate levels were significantly lower in the BHE rats than in the Wistar rats. Increases in total ketones, while at this age (50 days) not excessive (still being within the normal range), suggest that utilization of these metabolites by the peripheral tissues is not as great in the BHE rat compared to the Wistar rat. These differences suggest that BHE animals would have developed diabetes at a later age. In fact, their siblings who were maintained to the age of 300 days were indeed diabetic, with fasting glucose values ranging between 180 and 240 mg/100 ml. Normal fasting rats have glucose values of 80–90 mg/100 ml. Serum glycerol levels were higher in the BHE rats than in the Wistar rats and these findings are compatible with those reported by others (2).

[5] Purina Laboratory Animal Chow, Ralston Purina Co.

[6] Tobin, R. B., Berdanier, C. D., DeVore, V., and Ecklund, R. E., (manuscript submitted for publication).

differences in the serum glycerol
vere also observed in heparin-
animals infused with saline (Table
ese baseline values were higher
ose reported in Table I, but the
es in glycerol levels can be ex-
as being due to the effect of hepa-
poprotein lipase. When the values
I were subtracted from the initial
n Table II, it was apparent that
ere no differences between the
1 the effects of heparin on lipopro-
ise. That is, the strains did not
their heparin-induced lipoprotein
tivity.
rly, the two strains did not differ
clearance of the different doses of
The greater the dose of glycerol
ered, the longer the time required
lycerol level in the blood to return
einfusion level. At the 35- and 70-
ose levels, less than 30 min were
for the animals to return to their
on glycerol level. At the 140-
evel, however, it appeared that
n 30 min would be required before
the animals would return to their preinfusion level. These observations suggest that the high serum glycerol levels reported in Table I were not due to a decreased hepatic clearance of glycerol by BHE rats. In addition, the lack of strain differences in the clearance of glycerol from the blood indicates that in the young rat (75 days) the liver cell can rapidly adapt to changes in the concentration of this substrate and metabolize it rapidly.

This suggestion is supported by the lack of strain difference in the levels of glycerol in the liver tissue frozen immediately after the last blood sample was drawn. Serum glycerol levels were higher in the BHE rats infused with either 35 or 70 μmoles of glycerol/100 g body weight, but these differences were apparent even before the infusion began, whereas the animals infused with the highest glycerol levels had not yet recovered from this treatment. When the ratios of serum to liver glycerol levels were calculated (Table III), BHE rats had higher ratios than Wistar rats when they were infused with saline or 70 or 140 μmoles of glycerol. At the lowest glycerol dose, however, there were no strain differences in this ratio, although at this dose the ratios for BHE rats were much lower than for rats of the other treatment groups. Ratios higher than 1 indicate that more glycerol was found in the serum than in the liver tissue. Ratios less than 1 indicate that the glycerol in the serum had been rapidly transferred into the liver cell and metabolized. The glycerol could have been phosphorylated to α-glycerophosphate via glycerol kinase

E I. BLOOD METABOLITES IN NONFASTED BHE AND WISTAR RATS.

lites)	Strain	
	BHE	Wistar
	3.06 ± 0.29[a]	3.59 ± 0.23
	0.009 ± 0.005	0.090 ± 0.005
ybu-	0.298 ± 0.037	0.213 ± 0.013*
ate	0.019 ± 0.002	0.025 ± 0.002*
	0.089 ± 0.012	0.050 ± 0.005*

± SEM for 10 rats.
differences are significant ($P < 0.05$).

LE II. EFFECT OF VARYING INTRAVENOUS GLYCEROL INJECTIONS ON THE TRANSHEPATIC BLOOD GLYCEROL LEVELS

Minutes after glycerol						
-5	1	3	5	10	20	30
0.164 ± 0.034*	0.095 ± 0.032	0.072 ± 0.014	0.112 ± 0.014	0.126 ± 0.020	0.112 ± 0.028	0.121 ± 0.022
0.094 ± 0.030	1.000 ± 0.080	0.25 ± 0.048	0.155 ± 0.031	0.158 ± 0.004	0.102 ± 0.005	0.104 ± 0.007
0.202 ± 0.007	3.170 ± 0.700	0.369 ± 0.009	0.221 ± 0.006	0.183 ± 0.002	0.180 ± 0.004	0.207 ± 0.003
0.184 ± 0.003	7.280 ± 2.210	3.547 ± 0.395	1.500 ± 0.590	0.721 ± 0.300	0.465 ± 0.143	0.281 ± 0.009
0.244 ± 0.008*	0.144 ± 0.017	0.133 ± 0.010*	0.159 ± 0.16*	0.167 ± 0.015*	0.227 ± 0.031*	0.230 ± 0.034*
0.216 ± 0.090*	1.330 ± 0.220	0.279 ± 0.050	0.218 ± 0.003*	0.193 ± 0.002*	0.196 ± 0.002*	0.180 ± 0.044*
0.221 ± 0.064*	2.470 ± 0.620	0.910 ± 0.265	0.641 ± 0.249	0.293 ± 0.012*	0.237 ± 0.036*	0.428 ± 0.017*
0.235 ± 0.006*	5.740 ± 1.600	3.230 ± 0.440	1.950 ± 0.310	1.420 ± 0.630	0.378 ± 0.070	0.293 ± 0.008

icromoles per milliliter ± SEM for six rats.
fferences are significant ($P < 0.05$).

TABLE III. SERUM GLYCEROL, LIVER GLYCEROL, AND α-GLYCEROPHOSPHATE LEVELS 30 min AFTER INJECTION OF VARYING LOADS OF GLYCEROL.

Dose of glycerol (μmoles/ 100 g body wt)	Serum glycerol (μmoles/ml)	Liver glycerol (μmoles/g)	Serum/liver glycerol	Liver α-glycero-phosphate (μmoles/g)	α-glycer phate/gl
Wistar					
0	0.121 ± 0.022[a]	0.14 ± 0.04	0.85 ± 0.05	0.93 ± 0.28	6.57 ±
35	0.104 ± 0.007	0.21 ± 0.03	0.48 ± 0.04	1.24 ± 0.24	5.90 ±
70	0.207 ± 0.003	0.51 ± 0.31	0.41 ± 0.01	1.69 ± 0.85	3.31 ±
140	0.281 ± 0.009	0.24 ± 0.06	1.17 ± 0.06	1.10 ± 0.42	4.58 ±
BHE					
0	0.230 ± 0.034*	0.22 ± 0.03	1.05 ± 0.01*	1.10 ± 0.82	5.00 ±
35	0.180 ± 0.044*	0.42 ± 0.20	0.44 ± 0.05	1.21 ± 0.60	2.88 ±
70	0.428 ± 0.017*	0.34 ± 0.06	1.16 ± 0.03*	1.44 ± 0.71	3.88 ±
140	0.293 ± 0.008	0.25 ± 0.05	1.27 ± 0.02*	0.97 ± 0.56	4.24 ±

[a] Mean ± SEM for six rats.
* Strain differences are significant ($P < 0.05$).

or dehydrogenated to form glyceraldehyde through the action of aldose reductase. Usually, glycerol is phosphorylated rather than dehydrogenated since the K_m of glycerol kinase for glycerol is lower than the K_m of the aldose reductase for this metabolite (15). Glycerol kinase has been reported to be more active in BHE rats than in Wistar rats (2), but if this were true then one would expect BHE rats to have higher ratios of α-glycerophosphate to glycerol than Wistar rats. No strain differences in the amount of α-glycerophosphate were found, but when ratios of this metabolite to glycerol were calculated BHE rats had lower ratios, again suggesting that these rats probably did not have more glycerol kinase activity than Wistar rats. The lack of agreement between these findings and those reported by Gardner and Reiser (2) may have been due to the fact that an *in vitro* assay such as that used by Gardner and Reiser provides all the necessary substrates and cofactors in optimal amounts, amounts which may not be present *in vivo*. Thus, an increase in an *in vitro* enzyme activity may be an artifact of the measuring system. No strain differences were observed in the liver lipid content of the tissue powders. BHE livers contained 33.9 ± 8.5 mg of lipid/g of liver and Wistar livers contained 35.3 ± 8.5 mg of lipid/g of liver. Treatment differences were negligible. These observations are consistent with previous reports in that the strain differences in liver lipid do not appear until the animal is mature (more th days old) (1, 4).

In liver tissue from rats not subj glycerol infusion, heparin, or an no strain differences in hepatic g α-glycerophosphate, dihydroxyacetor phate, or 3-phosphoglycerate we served (Table IV). The strain dif in the ratio of α-glycerophosphate erol were again observed. BHE r lower ratios than Wistar rats, in a less active phosphorylation of gly α-glycerophosphate. At the sam these rats had higher phospho states than Wistar rats. The highe phorylation states were due to an in ATP and decreases in ADP an in BHE rats compared to Wistar rats

While no differences in the end α-glycerophosphate shuttle activiti observed, significant strain differe the activities of the complete shutt observed (Table V). BHE isolate chondria had less shuttle activity state 3 and state 4 conditions (without added ADP). While sign greater cytosolic α-glycerophosph hydrogenase activity has been repo 4) in BHE rats, the activity of th sponding enzyme in the mitochonc significantly less in this strain than Wistar strain (Table VI). These are compatible with the findings creased mitochondrial α-glyceroph shuttle activity in BHE rats.

Discussion. While the results

t study did not identify the source "extra" glycerol in the serum, they monstrate that the BHE liver, like istar liver, was capable of disposing e loads of free glycerol. The normal e of this metabolite has been re- to be approximately 11 min (16). udies confirm these findings; in the d 70-μmole groups the serum glyc- evels returned to their preinfusion within 20 min, with no significant changes in liver glycerol levels 30 min after the glycerol load.

From the results it is also clear that increasing the level of incoming glycerol to the liver did not increase the liver lipid content; findings which agree, in part, with those of Lin *et al.* (3). It has been reported that 400-day-old BHE rats had large quantities of hepatic glycerol (17). Apparently, in the BHE rat, glycerol accumulates in the liver because the means for its disposal are not fully active. This becomes apparent as the animal ages. Some evidence for this less active glycerol metabolism is seen in the strain differences in the ratios of serum to liver glycerol and liver α-glycerophosphate to glycerol (Tables III and IV). BHE rats had lower ratios of these liver metabolites than Wistar animals, indicating that in these rats the glycerokinase reaction was rate limiting. *In vitro* the enzyme has been shown to be more active in BHE rats than in Wistar rats (2). However, *in vitro* assays do not always replicate *in vivo* conditions and since the conversion of glycerol to α-glycerophosphate was less, one must assume that some regulatory factors are active *in vivo* and result in a less active enzyme. It has been reported that glycerol kinase activity is dependent on adequate levels of ATP (15, 18). Inspection of the data presented in Table IV shows that the BHE livers had greater quantities of ATP than Wistar livers. Similarly, glycerol ki-

TABLE IV. LIVER METABOLITES AND HORYLATION STATES IN NONFASTED BHE AND WISTAR RATS.

	Strain (μmoles/g of liver)	
	BHE	Wistar
l	46.4 ± 3.9[a]	45.2 ± 9.1
rophosphate	214 ± 52	260 ± 22
rophos-/glycerol	4.86 ± 0.28	7.13 ± 1.27*
xyacetone hate	50.1 ± 5.3	47.4 ± 2.8
hoglycerate	476 ± 32	406 ± 43
	2551 ± 80	2270 ± 50*
	1136 ± 45	1346 ± 26*
	246 ± 29	402 ± 19*
	4691 ± 412	4968 ± 465
ADP)	838 ± 80	596 ± 50*
$_4^{2-}$) cytosol		
ADP)	3.47 ± 0.53	2.10 ± 0.25*
$_4^{2-}$)[b] mito ×		

an ± SEM of eight rats.
culated from the values of the compartmented ites and redox state of each group (24).
ain differences are significant ($P < 0.05$).

BLE V. α-GLYCEROPHOSPHATE SHUTTLE ACTIVITY IN INTACT LIVER MITOCHONDRIA FROM BHE AND WISTAR RATS.

Micromoles of NAD produced per milligram of protein per minute			
Endogenous[a]		Complete[b]	
State 4 (−ADP)	State 3 (+ADP)[c]	State 4 (−ADP)	State 3 (+ADP)
1.24 ± 0.68 (8)[d]	0.84 ± 0.36 (8)	6.52 ± 0.58 (9)	5.14 ± 0.46 (9)*
1.38 ± 0.58 (8)	0.09 ± 0.35 (8)	12.64 ± 1.50 (9)**	9.96 ± 0.80 (9)*, **

tochondrial protein, 5 mg, was suspended in a medium containing 75 m*M* mannitol, 10 m*M* phosphate pH 7.4), 75 m*M* KCl, 5 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 7.4), 5 m*M* NADH, and water to a volume l. Flasks were incubated for 20 min at 37°.
complete system contained in addition to the above, 20 m*M* DL-α-glycerophosphate, 5 units of α-hosphate dehydrogenase, and 30 nmoles of rotenone/g of protein to a volume of 3 ml.
P, 4 m*M*, was added where indicated.
an ± SEM; number of samples in parentheses.
ferences in NAD production due to the addition of ADP significant ($P < 0.05$).
rain differences in NAD production are significant ($P < 0.001$).

TABLE VI. MITOCHONDRIAL α-GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY.

Strain	Enzyme activity (μmoles of O_2/mg of mitochondrial protein/min)
BHE	9.2 ± 1.1[a]
Wistar	12.0 ± 1.1*

[a] Mean ± SEM of eight rats per group.
* Strain differences are significant ($P < 0.05$).

nase can be inhibited by ADP (15); again, the data in Table IV would suggest that the enzyme is not so inhibited. Some inhibition of the enzyme might be anticipated if there were some strain differences in the distribution and exchange of adenine nucleotides. This does not appear likely though, in view of the fact that the ATP/ADP · HPO_4 ratios in both the cytosol and mitochondria are greater in BHE rats than in Wistar rats. If there were differences in adenine nucleotide exchange, then one might anticipate a discordance in the phosphorylation states of the two compartments. One cannot rule this out, however, until measures of adenine nucleotide translocase are made.

The liver cell actively transports glycerol into the cell (16, 18) to the extent that an equilibrium between the intracellular and extracellular compartments is seldom established. As glycerol is rapidly metabolized within the cell, more extracellular glycerol diffuses in. In Wistar rats, glycerol does not accumulate in either the serum or the liver and therefore one must assume that the accumulation seen in the BHE rat is due to some constraint in glycerol metabolism within the cell. Conceivably this constraint could include a less active glycerol kinase and/or a less active mitochondrial shuttle system.

Houstek *et al.* (20) have suggested that the α-glycerophosphate shuttle serves a regulatory function in the metabolism of α-glycerophosphate by brown adipose tissue. In liver, it may also serve to regulate metabolism by suppressing palmitylcarnitine conversion to palmityl CoA (21). When mitochondrial α-glycerophosphate dehydrogenase is low, the activity of the shuttle is low and β oxidation of fatty acids is not suppressed. This has a net effect of increasing the mitochondrial $NAD^+/NADH$ ratio as well as increasing the ATP/ADP ratio (21–23). Changes in the oxidation of palmitylcarnitine can affect the relative distribution of the adenine nucleotides within the cell through an inhibitory effect of acyl CoA on ATP translocase (23, 24). Any disruption of this transport process would be reflected in a lack of a coordination between the metabolic processes of the intra- and extramitochondrial compartments of the cell (23). While further work must be conducted in order to firmly establish in the BHE rat that such changes in metabolic regulation have occurred, the present data can be taken as strong suggestive evidence of such changes. Lower α-glycerophosphate shuttle activity has been shown (Table V), as have lower mitochondrial α-glycerophosphate dehydrogenase activity and higher phosphorylation states (Tables VI and IV). All of these observations are compatible with the previously reported decrease in energy utilization versus energy stored as fat (4).

Summary. The metabolism of glycerol has been studied in BHE and Wistar rats using measures of transhepatic glycerol disposal, assays of glycerol and glycerol metabolites in frozen clamped hepatic tissue, and through the study of mitochondrial α-glycerophosphate shuttle activity and α-glycerophosphate dehydrogenase activity. Strain differences in glycerol metabolism were observed, with the BHE rats having higher serum glycerol levels, lower presumed glycerolkinase activity, lower α-glycerophosphate shuttle activity, and lower mitochondrial α-glycerophosphate dehydrogenase activity. These findings were presumed to indicate a lack of coordination between the cellular compartments, explaining the previously reported enhanced hepatic lipogenesis seen in BHE rats.

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Postnatal Plasma Concentration and Urinary Excretion of Na and K in the F (39980)

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Introduction. A variety of studies has indicated that in neonatal animals, homeostatic responses resulting in increased excretion of salt and water do not readily occur. Human infants (1) as well as neonates of other species do not increase urinary salt or water excretion as much as adults following loading by water or saline (2–7). Young rats are unable to respond to blood volume expansion until they are between 35 and 40 days old (8). This age is close to that of 33 days when Stopp *et al.* found appropriately increased renal excretion of intraperitoneally administered saline (7). The absence of an increase in renal excretion following saline administration could reflect a change in absorption of the solution, but more recently, it has been established that the response to volume expansion produced by intravenous saline infusion is not as great in the neonate as it is in the adult for the rat (Misanko, Bengele, and Solomon, unpublished) and dog (5), indicating a lack of response by the kidney.

More recently, it has been shown that during postnatal development of the rat, tissue ionic contents change with a critical period (9) which also occurs at around 40 days (10). Relatively little work has been done on age-dependent changes in electrolyte balance and basal urinary excretion of electrolytes during postnatal development in the rat. To this end, both clearance and metabolic studies have been carried out to examine changes in plasma sodium and potassium concentration as well as urinary excretion of these ions during maturation.

Materials and methods. Animals. rats were used exclusively in this Usually, pregnant rats were obtain our commercial supplier (Simonsen, Calif.) and pups were born in our and then used at specified ages. Thre of 10 pups each were sent at 15 day after being born in California. All were kept in a temperature- and h controlled room with a photoperiod light and 12-h dark. Food and ta were available *ad libitum*. Pup weaned at 21 days of age.

Clearance studies. On the day o the animals were anesthetized an ances were carried out using stand cedures for this laboratory (8, 11). animals were anesthetized with inac jugular vein was cannulated for infu Ringers solution containing [^{3}H]in fusion rates were 0.5 ml/hr for weighing less than 100 g, and 1 m all other animals. The bladder was lated for collection of urine in plasti After at least 2 hr of equilibration, longer time when it was required fo zation of urine flow, we determine consecutive clearance studies of 20- ration, with blood samples being c at midperiod.

Urine volume was estimated as th of urine collected. Glomerular filtrat (GFR) was calculated as the clear inulin, the plasma and urine radi being measured by scintillation c Statistical analysis was carried out u paired *t* test. Some of the data r here have been taken from controls work from this laboratory. They ha reanalyzed to answer the questions ered in this paper.

Metabolic studies. Animals were for study at varying times after birt days before the studies, animals wer in metabolic cages and allowed to

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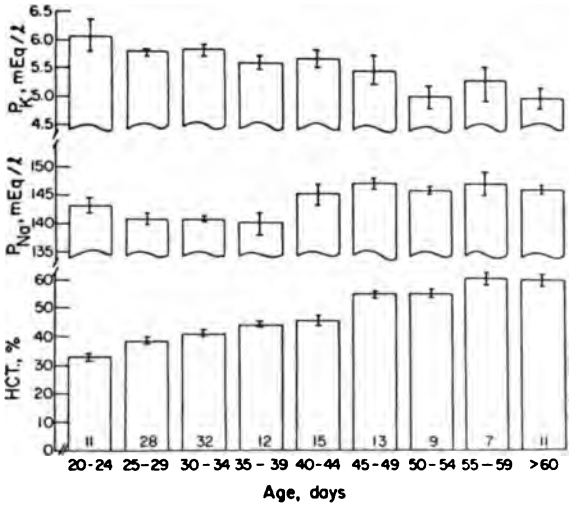
ıe metabolic cages were modified to a cup-within-a-cup of crushed rat Zia Brand Mouse and Diet, S.W. Son, Inc.) and a drinking cup of tap ırotected against contamination by a ıd kept at constant level by an exter- ter bottle. Twenty-four-hour food ıption, loss from the water bottle, ne output ($\dot{V}$), collected under min- , comprised the daily records main- on these rats. Urinary and plasma and potassium concentrations were ined by flame photometry. The ani- ı the metabolic studies, as well as ormally housed, were weighed daily.

!ts. Hematological values. Age-de- t hematological changes during de- ent of the rat are shown in Fig. 1. ıst striking observation is the change atocrit. This variable increases with t does not show a "critical period." solute values of hematocrit of mature ; are high as compared to other lab- ɛs. It should be remembered, how- ıat our studies are carried out at a evation and under semiarid condi- As a result, our control aimals are ly dehydrated by standards in other ories. Rather, it appears best fitted perbolic function. The change cannot ıted to a generalized reduction in llular fluid since the time course of changes in water content of various tissue is different than found here. Water content reaches a minimum at 20–25 days postnatally (10).

P_{Na} also shows an increase. In contrast to hematocrit, the increase is abrupt and shows a critical period between 40 and 45 days of age (Fig. 1). Before this time, average P_{Na} is between 140 and 143 meq/liter, while after 45 days it is 146–147. P_K tends to change in the opposite direction, but the changes are relatively small and continuous (Fig. 1). A significant difference ($P < 0.05$) exists between the youngest age group (20–24 days) and the over-60-day-old age groups. No critical period is evident.

Clearance studies. Renal functional changes are shown in Fig. 2. Glomerular filtration rate (GFR) does not show any marked age dependence in these studies when normalized per kilogram body weight, although we have previously observed a low GFR in our youngest animals (8, 11). Some differences do exist between individual groups (35–39 and 45–49 days old being lower than others). Since no trend can be seen, it would appear that these differences are spurious and probably result from preparative or other experimental errors. Marked changes are seen in $U_{Na}V$ and $U_K V$. $U_{Na}V$ is low until the 40- to 45-day critical period and then rises, while



. Changes in plasma Na and K and hematocrit with development of rat pups. Numbers at bottom of ndicate the number of rats in each group, here and in the following figures. Bars indicate SEM.

the opposite is true for U_KV. Because not all young groups show significant differences from all mature groups, we have analyzed the comparison of group means before 40 days of age as compared to after 45 days of age. The analysis is presented in Table I and indicates a highly significant difference in excretion of the two ions before and after the 40- to 45-day critical period.

Since both plasma concentrations and urinary excretions are changing during development, a better insight into the changes in renal function may be obtained by looking at fractional excretions of these ions. The individual age group data are shown in Fig. 3, and the group analysis is presented also in Table I. It is clear that a marked increase in FE_{Na} occurs at the 40- to 45-day critical period, while a decrease in FE_K takes place at the same time.

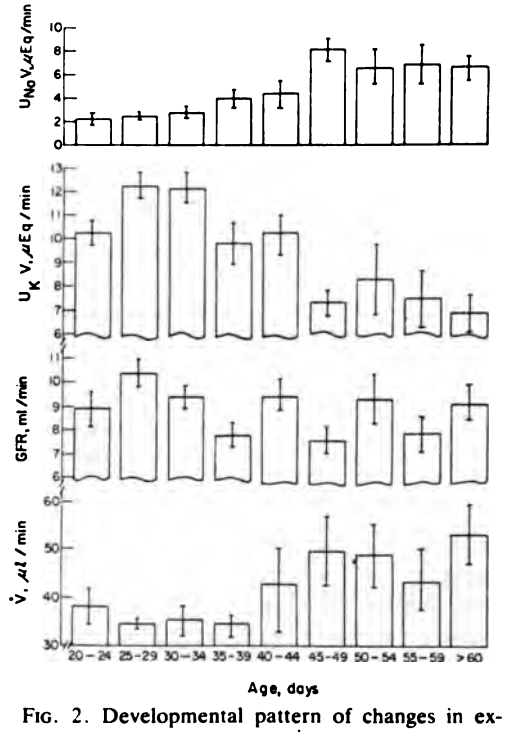


FIG. 2. Developmental pattern of changes in excretion of Na and K, and in $\dot{V}$, as well as changes in GFR. Bars show SEM. All data are normalized per kilogram of body weight.

Metabolic studies. Food intake, sodium, and potassium excretion are shown in Table II. As animals get older, food intake decreases when normalized for body weight. Water intake shows a parallel reduction. Renal excretion of both sodium and potassium decreases. The decrease, however, does not parallel the decrease in food intake. If the data are calculated as $U_{Na}V$ per gram of food ingested, there is an increase of about 20% between the youngest animals (27–30 days) and the animals 40 days of age (Table II). Thereafter, there is a second abrupt rise, with $U_{Na}V$ again doubling. A similar pattern is shown in excretion of potassium. The increase, however, in U_KV is less than half of the increase in $U_{Na}V$. Over the age range studied, U_KV always exceeds $U_{Na}V$. If, however, we examine the urinary Na/K ratio (Table II), we then find that up until 40 days postnatally it is constant, but then rises abruptly to a significantly higher level. These changes in Na/K are consistent with the changes in electrolyte excretion we observed in our clearance studies.

Discussion. These studies show that: (i) Both plasma sodium concentration and hematocrit increase as rat pups age; (ii) urinary electrolyte excretion during clearance studies also changes with age so that sodium excretion increases and potassium excretion decreases at the same "critical period," 40 days; (3) although absolute rates of Na and K excretion in metabolic studies are not like those in the clearance ratios, changes in Na/K excretion ratios

TABLE I. COMPARISON OF URINARY EXCRETION OF SODIUM AND POTASSIUM IN INFANT AND MATURE RATS.

	$U_{Na}V$[a]	FE_{Na}[b]	U_KV[a]	FE_K[b]
Infants	2.876 ± 0.398	0.249 ± 0.048	11.130 ± 0.637	22.2 ± 1.07
Mature	7.020 ± 0.354	0.655 ± 0.07	7.515 ± 0.296	17.0 ± 0.47
t	7.73	5.13	5.14	4.35
P	<0.001	<0.005	<0.005	<0.005

[a] $U_{Na}V$ and U_KV are expressed as milliequivalents per minute per kilogram body weight.
[b] FE_{Na} and FE_K are expressed as percentage of filtered load.

ire consistent with the results obtained in learance studies.

Although considerable work has been one on age-dependent changes in compo- ition of body fluids postnatally, we are ble to find but one report on develop- ıental changes in plasma electrolytes in ıe rat. Yunibhand and Held found a P_{Na} f 142.8 meq/kg of H_2O in 1-day-old ani- ıals after 24 hr of water deprivation (12). $_{Na}$ then fell to 136 meq/kg of H_2O at 15 ays, which was in turn succeeded by a ise which reached mature levels at 40 ays. Comparable postnatal increases in $_{Na}$ have been described for the mouse 13) over the first 40 days of life. In the og (14), there is a suggestion of a change

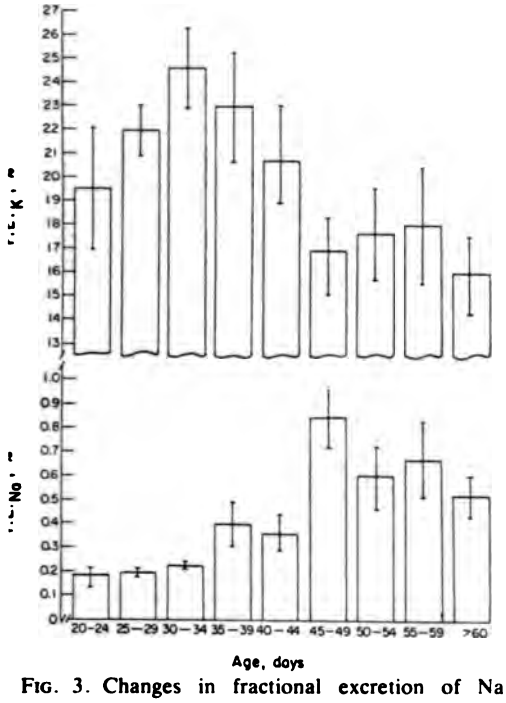


FIG. 3. Changes in fractional excretion of Na ıd K as rat pups grow older. Bars show SEM.

in P_{Na} during the first postnatal week, while in man no postnatal age-dependent changes in P_{Na} are evident (15). A marked change in P_{Na} is found only in rodents, suggesting a species dependence. There are fewer differences between laboratories in reports about postnatal changes in P_K. In man (15), dog (14), and rat (12), there is a decrease in P_K after birth, although the time courses are different. In the dog, the change occurs during the first postnatal week, while in the rat it has been reported to continue until animals are 20 days of age (12). This latter finding is inconsistent with the data reported here, in that our youngest animals (20–25 days old) had the highest P_K and the trend was for a continuous decrease over the full age range for study. In the mouse, the postnatal change in P_K is equivocal (16).

Kersten and Braünlich also found that sodium excretion increases with age or, as shown here (Fig. 2), in parallel with the change in control P_{Na}. In contrast to their studies, the excretion of K is reduced during development. In the study of Kersten and Braünlich (17), they found a minimum in K excretion at 15 days, with increasing rates of excretion as animals aged. The trend was not continuous, however, since 5-day-old rats had a high excretory rate. The results of these studies are in distinct contrast to ours. Since this study group also used Wistar rats, it is unlikely that this contradiction is a result of differences in rat strains. Our own metabolic results show changes in electrolyte excretion which are consistent with the excretion data obtained during clearance studies, so that one cannot account for the discrepancy on the basis that Kersten and Braünlich carried out their studies in metabolic cages.

TABLE II. FOOD INTAKE AND ELECTROLYTE EXCRETION OF CONTROL RATS DURING GROWTH.

Age (days)	N	Food intake (g/day/kg)	Water intake (ml/day/kg)	$U_{Na}V$ (meq/day/kg)	U_KV (meq/day/kg)	Na/K
<30	22	16.1 ± 0.28	31.2 ± 1.3	18.91 ± 1.03	24.48 ± 0.82	0.773 ± 0.030
31-35	26	14.1 ± 0.42	24.6 ± 0.97	18.42 ± 0.43	23.78 ± 0.49	0.776 ± 0.013
36-40	24	12.1 ± 0.18	21.8 ± 1.15	15.67 ± 0.35	20.12 ± 0.45	0.781 ± 0.012
41-45	26	11.0 ± 0.17	18.5 ± 0.40	15.30 ± 0.45	18.24 ± 0.43	0.840 ± 0.016
46-50	20	9.7 ± 0.19	17.3 ± 0.87	14.24 ± 0.31	16.92 ± 0.38	0.844 ± 0.016
51-55	10	9.2 ± 0.16	14.1 ± 0.36	14.15 ± 0.42	16.67 ± 0.56	0.850 ± 0.014

Other possibilities which could play a role are differences in route of fluid administration, strain, and food availability (18). Unless animals are grown under identical conditions, and are of the same strain and on the same diet, such differences as have been found may occur.

The question arises as to biological significance of the changes in electrolyte balance. If one examines the growth curves of rats in our laboratory, we get curves which are similar to those previously described by Widdowson and McCance (19). A striking feature of these curves is that there are two inflection points in the curve: one at about weaning, when there is an acceleration of absolute growth, and the other at about 40 days, when absolute growth rate reduces. Figure 4 shows the absolute growth rates for animals in our laboratory. Since food intake shows a continuous decline with age in our metabolic studies, one can hypothesize that one contributing factor is the need for sodium retention to accommodate the period of rapid absolute growth.

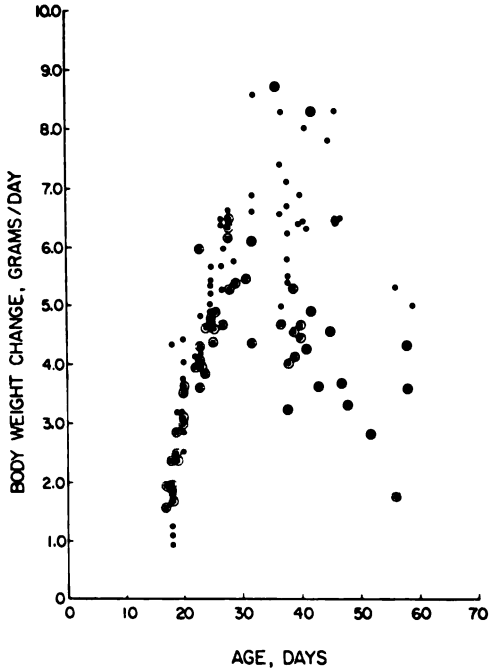


FIG. 4. Daily growth rates of rats as a function of age. Solid points show males; females are indicated by circled points.

Another factor which may be of significance is that tissue K relative to Na increases to 40 days of age, suggesting a maturation of the regulation of cellular to extracellular ionic ratios as has been found for muscle and brain (10, 20). Intracellular K increases in these tissues during this time period. No comparable studies are known for the kidney. Relatively more ingested K may be retained intracellularly in the mature to account for the decrease in K excretion.

One consequence of the relatively low plasma and $U_{Na}V$ is that the low plasma K may signal the kidney to conserve sodium. Such a signal may override the renal responses to blood volume expansion or saline loading and thereby be partially responsible for the inability of immature rats to respond appropriately to these stimuli (4, 8).

Summary. Rats show a critical period in electrolyte balance at around 40 days of age. P_{Na} and $U_{Na}V$ increase abruptly and $U_K V$ falls at this time. P_K shows a continuous decrease over the age ranges studied. Hematocrit shows a hyperbolic increase. In metabolic studies, urinary Na/K shows a significant increase at the same critical period.

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Attenuation of a β-Adrenergic Response in Rats Treated Chronically with Ethynyl Estradiol[1] (39981)

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Chronic administration of combinations of the estrogenic agent, ethynyl estradiol, and the progestational agent, norethynodrel, to intact female rats reduced their responsiveness to the β-adrenergic agonist, isoproterenol. In these studies responsiveness was assessed by measurement of the increase in heart rate and water intake following acute administration of isoproterenol (1, 2). An additional test that has also been used to assess β-adrenergic responsiveness is the increase in tail skin temperature following acute administration of isoproterenol (3).

The objective of the present experiment was to use this test to study the responsiveness of tail skin temperature to acute administration of graded doses of isoproterenol to ovariectomized rats treated chronically with several different doses of ethynyl estradiol.

Methods. Twenty-four female rats of the Blue-Spruce Farms (Sprague-Dawley) strain weighing 210 to 260 g were used. They were kept in hanging, galvanized mesh-wire cages in an animal room maintained at 24 ± 1° and illuminated from 0600 to 1800 hr. All rats were ovariectomized while anesthetized with sodium pentobarbital (40 mg/kg body weight) to avoid any contribution of endogenously produced ovarian hormones to the observed results. One week after ovariectomy, a Silastic tube containing crystalline ethynyl estradiol was implanted subcutaneously between the shoulder blades. Although it was originally intended that there should be three different doses of ethynyl estradiol, the first 12 rats were implanted in error with the same Silastic tubing (No. 602-281, 0.0315-in. wall thickness, 10 mm long). A second group of six rats was also implanted with Silastic tubing (No. 602-231, 0.0095-in. wall thickness, 10 mm long). The remaining six rats were implanted with Silastic tubing (602-231) which contained no steroid, and served as the control group. Prior to implantation, the sealed tubes were placed in a vacuum desiccator for 48 to 72 hr and weighed on an analytical balance. The tube in each rat was palpated at weekly intervals to be certain it was still in place.

Dimethylpolysiloxane (Silastic) tubing has been shown to allow diffusion of certain crystalline steroids into various media at a constant rate over relatively long periods of time (4, 5). Previous experience in this laboratory has indicated that this method of steroid administration provides a reliable means of achieving reasonably constant drug release for periods of up to 6 months.

During the fifth and sixth weeks after implantation of the Silastic tubes, each rat was lightly restrained in a Lucite, tunnel-type cage large enough to hold it comfortably while preventing it from turning from head to tail. The cages were provided with access ports through which the rat could be injected during the experiment without removing it from the cage. A copper-constantan thermocouple was inserted 5 cm into the colon and held in place by a piece of adhesive tape which bound it to the tail. The temperature of the dorsal surface of the skin of the tail; approximately 2 cm from its base, was measured by a second copper-constantan thermocouple. This thermocouple was held in place by weaving it into a single layer of gauze sponge 3 cm long. The two ends of the sponge were bound to the tail with adhesive tape. The thermocouples were led off to a recording potentiometer which recorded the skin and

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colonic temperatures of each rat at 1-min intervals. After 30 min of restraint in a room maintained at 24 ± 1°, each rat was injected sc with either 50, 100, or 200 μg of *l*-isoproterenol (Isuprel hydrochloride, Winthrop Laboratories, New York)/kg body weight. Measurements of colonic and tail skin temperatures continued for an additional 2 hr, after which the rats were returned to their stock cages. Three to five days were allowed between studies.

Statistical analyses of the data were made by means of an analysis of variance (6). Significance was set at the 95% confidence limit.

At the end of the experiment, the tubes containing ethynyl estradiol were removed from their subcutaneous sites and dried for 48 hr in a vacuum desiccator. The tubes were then weighed on an analytical balance and daily drug dose was calculated from the weight loss of the tube and the mean body weight of the rat during the period of drug administration. The tubes that were removed never contained fluid inside them.

Results. When the control rats were administered 50 μg of isoproterenol/kg sc, tail skin temperature increased from 25 to 31.6° within 30 min (Fig. 1A). An increase in tail skin temperature was detectable within 10 min after drug administration. Tail skin temperature returned to control level within 100 min after administration of isoproterenol. No change in tail skin temperature was seen in either of the ethynyl estradiol-treated groups in response to this dose of isoproterenol. Initial colonic temperatures of both groups treated with ethynyl estradiol were significantly ($P < 0.05$) less than the temperature of the control group prior to administration of isoproterenol. Colonic temperatures of all three groups fell during the course of the experiment (Fig. 1B). However, the colonic temperatures of both ethynyl estradiol-treated groups remained significantly lower ($P < 0.05$) than the temperatures of the control group following administration of isoproterenol.

Administration of 100 μg of isoproterenol/kg sc was accompanied by an increase in tail skin temperature from 24.8 to 32.0° within 30 min (Fig. 2A). The time course of change in tail skin temperature for the

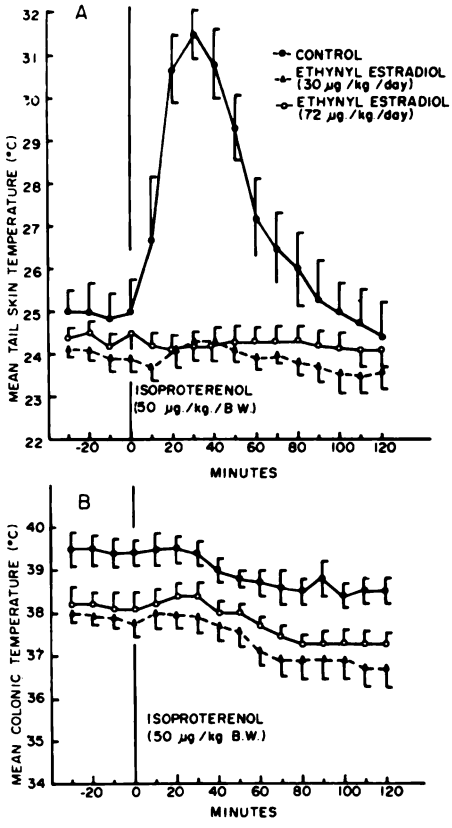


FIG. 1. (A) Mean tail skin temperatures of control and ethynyl estradiol-treated groups prior to, and following, administration of 50 μg of *l*-isoproterenol/kg body weight, sc, at time 0. Designation of each group is shown in the figure. One standard error is set off at the means. (B) Mean colonic temperatures of the same three groups during the same experiment are shown.

control group was very similar to that described above for the lower dose of isoproterenol, although tail skin temperature returned to control level only after 120 min following drug administration. In the case of the ethynyl estradiol-treated groups, the higher dose of isoproterenol used in this study increased tail skin temperature 2.8° in the group receiving the lower dose of the estrogenic agent and 1.1° in the group receiving the higher dose (Fig. 2A). Colonic temperatures of the three groups decreased during the course of the experiment (Fig. 2B). The initial colonic temperatures of the group receiving the lower dose of ethynyl estradiol were significantly lower than those

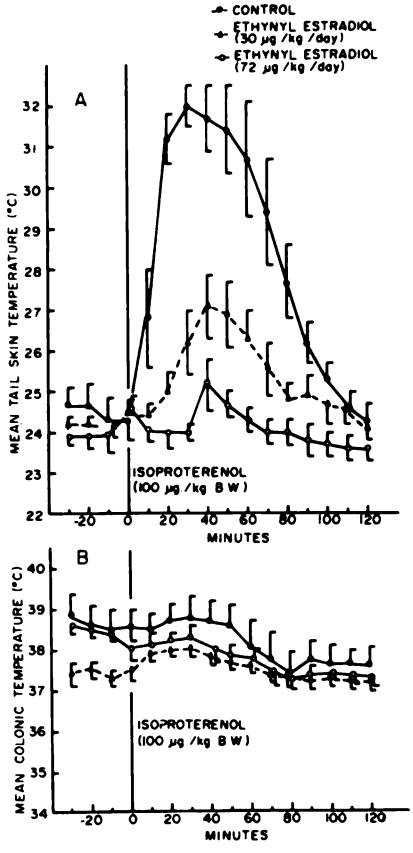


FIG. 2. (A) Mean tail skin temperatures of control and ethynyl estradiol-treated groups prior to, and following, administration of 100 μg of *l*-isoproterenol/kg body weight, sc, at time 0. Designation of each group is shown in the figure. One standard error is set off at the means. (B) Mean colonic temperatures of the same three groups during the same experiment are shown.

of the control group. All three treated groups showed changes in colonic temperature that approximated the time course of changes in tail skin temperature.

Administration of 200 μg of isoproterenol/kg to control rats was accompanied by an elevation of tail skin temperature from 24 to 32° (Fig. 3A). The maximal temperature was not different from that observed after administration of the two lower doses but was maintained for a longer period. Administration of this dose of isoproterenol to the ethynyl estradiol-treated groups resulted in an increase in tail skin temperature that was much attenuated compared to that of the control group (Fig. 3A). The group receiving the lower dose of ethynyl estradiol had an increase in tail skin temperature of 3.0°, while the group receiving the higher dose had an increase of 2.0°. Colonic temperatures of all three groups decreased during the course of the experiment (Fig. 3B). Both treated groups had lower colonic temperatures than controls during the control period. The lower colonic temperatures were maintained throughout the experiment.

The area under the curve of tail skin temperature versus time was determined by integration for each rat. The baseline value about which the integration was computed was the tail skin temperature at time 0. Any temperatures below this value were

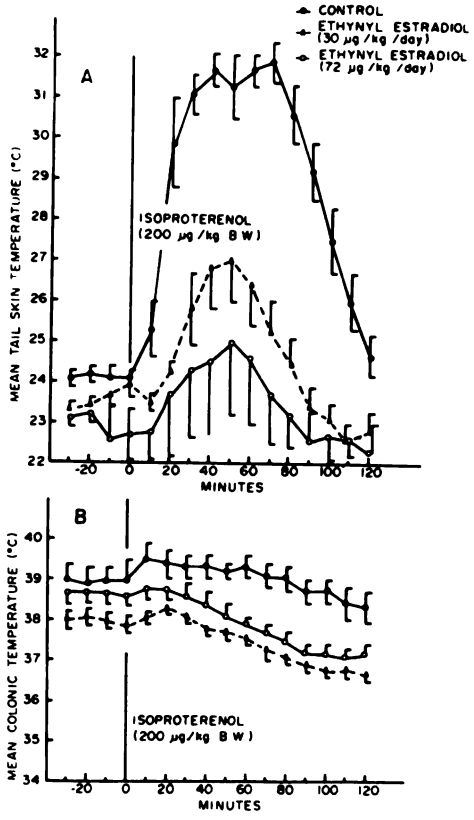
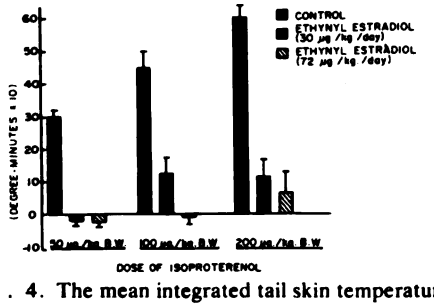


FIG. 3. (A) Mean tail skin temperatures of control and ethynyl estradiol-treated groups prior to, and following, administration of 200 μg of *l*-isoproterenol/kg body weight, sc, at time 0. Designation of each group is shown in the figure. One standard error is set off at the means. (B) Mean colonic temperatures of the same three groups during the same experiment are shown.

dered negative, while those above considered positive. The algebraic sum e integration over time is defined as ntegrated tail skin temperature. The ı integrated tail skin temperature of ontrol group increased with increasing ; of isoproterenol (Fig. 4). The inte- d tail skin temperatures of the two ed groups at each dose of isoproterenol l to differ from one another, but each ignificantly less ($P < 0.01$) than that e control group. Apparently the atten- n of responsiveness to administration proterenol was near its maximal level e group receiving the lower dose of yl estradiol.

the end of the 17th week of the iment, each rat was anesthetized with and the tube containing the drug was ved from its subcutaneous site. After ing, drying, and weighing each tube as ibed above, the group receiving the er-walled tube had a mean weight loss 3 ± 0.7 μg/day, while the mean weight of the thinner-walled tube group was ± 0.5 μg/day. When calculated on the of mean body weight during the exper- t, weight losses of 30 and 72 μg of yl estradiol/kg/day occurred from the

cussion. The tail of the rat, like the of the rabbit, appears to act as a al radiator. When the body of the rat eatened with overheating, tail blood tail skin temperature, and heat loss ase in an effort to dissipate the excess (7). Acute administration of the β-



. 4. The mean integrated tail skin temperatures under the curves in Figs. 1-3) for control and l estradiol-treated groups following administra- 50, 100, and 200 μg/kg of *l*-isoproterenol are . The groups are designated in the figure. One rd error is set off at the means.

adrenergic agonist, isoproterenol, produced a striking elevation in tail skin temperature of rats (Figs. 1-3) (8, 9). Whether this response is due to a direct action of the β-adrenergic agonist on tail vasculature or indirect via effects on metabolism is not as yet clear. It is clear, however, that this response is not produced by administration of isotonic saline (9). Furthermore, the response to isoproterenol can be blocked by the β-adrenergic antagonist, propranolol (9), and attenuated by chronic treatment with ethynyl estradiol (Fig. 4). Other studies have shown that tail skin temperature is not affected by the α-adrenergic agonist, phenylephrine (9). The time course for development of the attenuation was not studied here but was studied earlier in rats receiving combinations of ethynyl estradiol and norethynodrel (3). Reduced β-adrenergic responsiveness appeared from 3 to 5 weeks after initiation of steroid treatment and was present in all treated groups at 5 weeks (3). However, the earlier results may not be applicable here because of differences in experimental protocol. Earlier studies used different combinations of norethynodrel and ethynyl estradiol and the rats were not ovariectomized.

In all three studies described here, the control rats had initial colonic temperatures that were greater than those of the treated groups. The reduced colonic temperature of the treated rats suggests that the ability either to produce heat or to conserve it, or both, was affected by chronic treatment with ethynyl estradiol. The lower colonic temperatures of the treated groups may reflect a reduction in the contribution of the β-adrenergic system to maintenance of metabolic rate (heat production) under normal conditions. The ability of estrogen-treated rats both to produce and to conserve heat is currently under study in this laboratory.

The results of the present study do not provide clues regarding the mechanisms by which chronic administration of ethynyl estradiol attenuates the response to acute administration of isoproterenol. Whether one of these factors is a reduced responsiveness of the β-adrenergic receptor, or a site beyond, is also not clear at present and will require additional studies.

Summary. Chronic treatment of ovariectomized female rats with ethynyl estradiol (30 and 72 μg/kg/day) attenuated the increase in tail skin temperature characteristically accompanying sc administration of *l*-isoproterenol (50, 100, or 200 μg/kg). The mechanism by which the attenuation occurs awaits additional studies.

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An *in Vitro* Study of Pancreatic Ductal Cells[1] (39982)

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ction. The exocrine pancreas is
ito acinar cells which synthesize
te digestive enzymes and ductal
h secrete water and electrolytes.
vledge of acinar cell function has
tremendously during the past 10
ause techniques are available for
udies of acinar cells as a part of
pancreas, as slices, as isolated
s isolated cells, or as subcellular
On the other hand, our knowl-
luctal cell function has remained
static. Possible impediments to
ion of knowledge concerning duc-
unction are: (i) the complexities
n the isolation of ductal cells from
ng connective and acinar tissue;
nall number of ductal cells com-
acinar cells; and (iii) the efforts
to obtain sufficient amounts of
sue for study. Thus, it is not sur-
at most of our information con-
uctal cell function has been derived
siologic studies of water and elec-
retion utilizing the intact organ *in*
perfused organ *in vitro*.
cells have been well characterized
gically and histochemically (1-8).
n *et al.* have shown that the epithe-
of intermediate and large size
ated by dissection was rich in a
f enzymes (9). However, extensive
or biochemical characterization
cells has not been accomplished.
, there is no information to indi-
ts of fasting, feeding, or gastroin-
ormones on ductal cell function *in*

This paper reports studies on intact pancreatic ducts isolated by quick dissection and incubated under appropriate *in vitro* conditions. Fasting for 72 hr was associated with decreases in [^{14}C]glucose oxidation to $^{14}CO_2$ and alkaline phosphatase activity. There were no changes in the following: protein, RNA, or DNA content; ^{14}C-labeled amino acid incorporation into protein; [^{14}C]glucose or [^{14}C]glucosamine incorporation into glycoproteins; Mg^{2+}-ATPase, HCO_3^--ATPase, 5'-nucleotidase, or leucine aminopeptidase activities.

Methods. Male Sprague-Dawley rats (initial weights 300-350 g) housed in temperature-controlled quarters were used for all studies. Animals referred to as "fasted" were denied food for 72 hr; rats referred to as "fed" had free access to Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.). All rats were allowed free access to tap water.

The following materials were used: D-[U-^{14}C]glucosamine hydrochloride (237.7 mCi/mmole), D-[1-^{14}C]glucose (8.72 mCi/mmole), and L-[U-^{14}C]-labeled amino acid mixture (New England Nuclear Corp., Boston, Mass.); materials for incubation media, NCTC-109, nonessential amino acids (Microbiological Associates, Inc., Bethesda, Md.); calf thymus DNA and D-ribose (Nutritional Biochemical Corp., Cleveland, Ohio); orcinol (Fisher Scientific Co., Pittsburg, Pa.); diphenylamine (Eastman Organic Chemicals Division, Eastman Kodak Co., Rochester, N.Y.); bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.); hydroxide of hyamine 10-X, 1 *M* solution in methanol (Packard Instrument Co. Inc., Downers Grove, Ill.). Reagent kits for alkaline phosphatase (Cat. No. 15990) and leucine aminopeptidase (Cat. No. 15952) were obtained from Boehringer Mannheim Corp., New York.

Treatment of tissue. Rats were killed by

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cervical dislocation. The pancreatic duct was located, and acinar tissue was trimmed away with sharp microsurgical scissors. Approximately 4 cm of duodenum were left attached to the duct to facilitate handling; however, after acinar tissue was removed, the pancreatic duct was transected and the duodenum discarded. The pancreatic duct was placed on filter paper and kept moist with cold saline. Residual acinar tissue was removed by dissection and scraping with the blunt side of scissors. The duct was then cut longitudinally for maximal exposure to the incubation medium. The isolation procedure required 20 min for 12 ducts which were pooled, weighed, and placed in a flask containing freshly oxygenated tissue culture medium.

Studies of total protein, RNA, and DNA. Pooled ducts were weighed and homogenized in a ground-glass homogenizer using cold perchloric acid (PCA). The homogenate was centrifuged and the pellet washed twice with cold PCA. Methods used for separation of RNA, DNA, and protein were modifications of methods developed by Schmidt and Thannhauser (10) and Schneider (11). RNA was determined by the orcinol method using ribose as the standard (12, 13). DNA was determined by the diphenylamine method using calf thymus DNA as a standard (12, 13). Protein was determined by the method of Lowry *et al.* using bovine serum albumin as the standard (14).

Studies of ^{14}C-labeled mixed amino acid incorporation into protein. The pooled ducts were incubated for 30 min in a 25-ml Erlenmeyer flask containing 1 ml of incubation medium with 0.2 μCi of ^{14}C-labeled mixed amino acids. Methods used for *in vitro* incubation and determination of radioactive incorporation have been described (15, 16). Radioactivity was measured in a Packard liquid scintillation counter using a phosphor developed by Patterson and Greene (17).

Studies of [^{14}C]glucose oxidation. Pooled ducts were incubated in a 25-ml Erlenmeyer flask modified to contain a center well. Each flask contained 6.94 μmoles (294,000 cpm) of [^{14}C]glucose and 1 ml of incubation medium. Incubations were performed for 60 min at 37° in a shaking water bath. These methods were described pr (18).

Studies of [^{14}C]glucose and [cosamine incorporation into protein. ducts were incubated in a 25-ml Erl flask containing 1 ml of incubation with 0.05 μCi of [^{14}C]glucose or 1 [^{14}C]glucosamine for 60 and 30 min tively. Glycoproteins were precipita PCA and dissolved in KOH, and n ments were performed as describ 19).

Studies of ductal enzymes. Pool were homogenized with a ground-g mogenizer in 2 ml of cold buffer co 0.25 *M* sucrose and 10 m*M* trie mine-HCl, pH 7.4. An aliquot of mogenate was taken for measure DNA and protein. Mg^{2+}-ATPase sayed by the method used by Sim and Wizemann *et al.* (20, 9); HCO ase by the method of Simon *et al.* nucleotidase by the method of Di Purdom (21). Alkaline phosphatas sayed by the method of Hausam (22); leucine aminopeptidase by the of Nagel *et al.* (23). Reagent kits assays were obtained from the Bo Mannheim Corporation. Enzymes sayed under conditions which ga results with respect to both qu tissue and time of incubation.

Results. Table I shows proteir and DNA content of pancreatic du fed and fasted rats. The data are e in terms of tissue wet weight or mi of DNA (mean ± SE). There significant differences between fasted animals.

Table II shows values for inco of ^{14}C-labeled mixed amino acids teins by pancreatic ducts from fed animals. The results are expressed per minute incorporated per mill protein or as counts per minute rated into protein per 100 micro DNA. There were no significant di between fed and fasted rats.

Table III shows values for [^{14}C oxidation to $^{14}CO_2$. The results pressed as nanomoles of [^{14}C]glu dized per milligram of protein or micrograms of DNA. There were s

TABLE I. EFFECTS OF FEEDING AND FASTING ON PROTEIN, RNA, AND DNA.

	Protein		RNA		DNA
	μg/mg tissue wet wt	μg/100 μg of DNA	μg/mg tissue wet wt	μg/100 μg of DNA	μg/mg tissue wet wt
Fed	66 ± 4[a]	830 ± 78	0.55 ± 0.44	10.3 ± 1.2	7.92 ± 0.27
Fasted	65 ± 5	830 ± 49	0.49 ± 0.05	9.1 ± 1.2	7.91 ± 0.21

[a] Values are means ± SE of three experiments.

TABLE II. ^{14}C-LABELED MIXED AMINO ACID INCORPORATION INTO PROTEIN.[a]

	cpm/milligram of protein	cpm/100 μg of DNA
Fed	60.7 ± 15.9[b]	23.7 ± 2.5
Fasted	60.0 ± 8.3	20.3 ± 2.3

[a] Time of incubation, 30 min.
[b] Values are means ± SE of three experiments.

TABLE III. [^{14}C]GLUCOSE OXIDATION.[a]

	Nanomoles/mg of protein	Nanomoles/100 μg of DNA
Fed	$1.48 \times 10^{-2} \pm 0.35$*,[b]	$9.7 \times 10^{-3} \pm 1.0$**
Fasted	$0.46 \times 10^{-2} \pm 0.11$	$3.2 \times 10^{-3} \pm 0.5$

[a] Time of incubation, 60 min.
[b] Values are means ± SE of seven experiments.
* $P < 0.05$.
** $P < 0.005$.

decreases in [^{14}C]glucose oxidation with fasting when the data were expressed in terms of protein ($P < 0.05$) or DNA ($P < 0.005$).

Table IV shows values for [^{14}C]glucose or [^{14}C]glucosamine incorporation into glycoproteins. There were no differences between fed and fasted rats.

Table V shows mean values for Mg^{2+}-ATPase, HCO_3^--ATPase, 5'-nucleotidase, leucine aminopeptidase, and alkaline phosphatase activities expressed as a function of protein or DNA. No significant differences were found in Mg^{2+}-ATPase, HCO_3^--ATPase, 5'-nucleotidase, or leucine aminopeptidase activities.

Alkaline phosphatase activity in ductal tissue from fasted animals was significantly lower than in fed animals. Values for alkaline phosphatase activity in pancreatic juice from fed and fasted animals were essentially similar. Moreover, there was no difference between fed and fasted rats in total alkaline phosphatase activity of the whole pancreas. We concluded that the decrease associated

TABLE IV. INCORPORATION OF [^{14}C]GLUCOSE AND [^{14}C]GLUCOSAMINE.

	[^{14}C]Glucose[a]		[^{14}C]Glucosamine[b]	
	cpm/mg of protein	cpm/100 μg of DNA	cpm/mg of protein	cpm/100 μg of DNA
Fed	118 ± 8[c]	90 ± 7	249 ± 8	215 ± 15
Fasted	112 ± 8	86 ± 11	244 ± 11	208 ± 24

[a] Time of incubation, 60 min.
[b] Time of incubation, 30 min.
[c] Values are means ± SE of four experiments.

with fasting resulted from changes in the ductal epithelium where alkaline phosphatase activity is mainly located.

Studies were conducted to determine the degree of contamination of ductal tissue with acinar tissue. Morphologic examination using light microscopy revealed no acinar elements in the pellets of ductal tissue. Measurements of amylase activity in ductal tissue isolated from fed or fasted animals were essentially similar (0.385 ± 0.58 and 0.355 ± 0.39 units/mg tissue wet weight). The results of morphologic studies, the paucity of amylase activity in ductal tissue (<1% of amylase activity in acinar tissue per milligram tissue wet weight), and the lack of differences between fed and fasted animals would indicate that little or no acinar tissue contaminated the ductal preparation.

Discussion. These studies grew out of efforts to obtain more definitive information of a biochemical nature regarding the function of pancreatic ductal epithelium. Such information is particularly relevant since it is recognized that pancreatic ductal epithelium may be the primary site of origin for pancreatic adenocarcinoma.

The fed-fasted model was used to determine if there were differences in metabolic activities of pancreatic ductal epithelium

TABLE V

Enzyme	No. of experiments	μmoles of phosphate/hr/mg of protein		μmoles of phosphate/hr/100 μg of DNA	
		Fed	Fasted	Fed	Fasted
Mg^{2+}-ATPase	3	16.2 ± 6.7[a]	16.0 ± 6.9	19.4 ± 7.3	18.6 ± 6.9
HCO_3^--ATPase	3	15.1 ± 5.7	15.5 ± 4.6	17.5 ± 6.3	17.6 ± 5.8
5′-Nucleotidase	7	5.0 ± 0.6	5.0 ± 0.6	8.9 ± 1.3	8.5 ± 0.8
		μmoles/hr/mg of protein		μmoles/hr/100 μg of DNA	
Leucine aminopeptidase	7	0.06 ± 0.01	0.06 ± 0.01	0.109 ± 0.2	0.103 ± 0.2
Alkaline phosphatase	7	9.74 ± 1.27	6.14 ± 0.62*	16.4 ± 1.4	11.9 ± 1.1**

[a] Values are means ± SE.
* $P < 0.005$.
** $P < 0.05$.

with decidedly different states of nutrition. We have shown in earlier works using acinar tissue that fasting was associated with decreases in protein synthesis, specifically amylase, RNA synthesis, activities of polymerases I and II enzymes, as well as decreases in [^{14}C]glucose oxidation to $^{14}CO_2$ and [^{14}C]palmitate oxidation and incorporation into phospholipids. In addition, we demonstrated that increases observed in metabolic activities with refeeding were simulated by the administration of cholecystokinin-pancreozymin (16, 18, 24, 25). These observations have led us to conclude that many of the changes observed in the exocrine pancreas with feeding were under the control of gastrointestinal hormones (26).

The studies presented here show no differences between fed and fasted animals in protein content, RNA content, amino acid incorporation into protein, or amounts of [^{14}C]glucose or [^{14}C]glucosamine incorporation into glycoproteins. Electron microscopic studies of ductal epithelium have shown little endoplasmic reticulum or evidences of active protein synthesis (1, 2). Thus, we did not anticipate great changes in rates of protein or RNA synthesis. Since the duct epithelial cells in rat, unlike those in mouse and hamster, did not appear to be active in mucin secretion (27), we did not anticipate changes in glycoprotein synthesis.

Significant decreases in glucose oxidation with fasting support other observations that ductal epithelium has an active metabolic rate (7). The decreased glucose oxidation suggests that such functions may be under hormonal control.

We selected the five enzymes under study because of their possible role in secretory or transport activities. Bicarbonate-ATPase was shown to be localized to both plasma membrane and mitochondria in the pancreas (20). Wizemann found greater amounts of HCO_3^--ATPase in the small duct system, whereas Mg^{2+}-ATPase was significantly higher in large ducts, i.e., 700 to 1000 μm (9). 5′-Nucleotidase and leucine aminopeptidase were selected because of reported presence in ductal structures (9, 28). Alkaline phosphatase was demonstrated histochemically in ductal and transport epithelium (29–31).

The decreases in alkaline phosphatase activity in ductal tissue with fasting were of interest. Alkaline phosphatase activity was demonstrated in large ducts (700–1000 μm) and in small ducts (25–60 μm) by Wizemann *et al.* (9). Amounts of alkaline phosphatase activity in main ducts were 2.6 times greater than amounts in the whole pancreas. We found no differences in alkaline phosphatase activity with fasting or feeding in whole pancreas or in pancreatic juice.

We did not find differences with feeding or fasting in Mg^{2+}-ATPase, HCO_3^--ATPase, 5′-nucleotidase, or leucine aminopeptidase activities. Similar levels of activity with fasting or feeding would suggest that these enzymes were not inducible and, therefore, were not under control of gastrointestinal hormones.

Considerable technological difficulties were encountered in efforts to isolate and study pancreatic duct cells. At present, there appears to be two methodologic approaches to the isolation of ductal cells from acinar tissue. The first depends on the dissociation of cells by the use of collagenase

zymes and the separation by the
ity gradient techniques (32–34).
l method depends on the separa-
tal and acinar tissue by the use
ical methods. The techniques
s study, as well as the study by
et al., depended on the mechan-
al of acinar tissue (9).
used methods for cellular disso-
veloped by Amsterdam and Ja-
, 33). We then applied the dis-
lls to discontinuous Ficoll gra-
rding to the method of Spalsbury
r on albumin gradients according
hod of Sykes *et al.* (35). To date,
en unable to satisfactorily sepa-
cells from acinar cells using such
In the course of the dissociation
observed that larger and smaller
ined intact and could be studied

ies were expected in the separa-
tal, centroacinar, and acinar cells
l cells represent less than 5–8%
cellular mass (36). Nevertheless,
dies of ductal cells will be neces-
better understanding of mecha-
ter and electrolyte secretion and
changes associated with such

. Although pancreatic duct cells
characterized morphologically
hemically, information on their
y and mechanism is lacking. The
present work was to study se-
abolic parameters in the pan-
t of fed and fasted rats. The
was quickly isolated by dissection
d longitudinally. Twelve ducts
d for each study. Fasting for 72
associated with decreases in
e oxidation to $^{14}CO_2$ and alkaline
e activity. There were no
h fasting in the following param-
in, RNA, or DNA content; ^{14}C-
ino acid incorporation into pro-
glucose or [^{14}C]glucosamine in-
into glycoproteins; Mg^{2+}-ATP-
$^{-}$-ATPase, 5′-nucleotidase, or
inopeptidase activities. In con-
nar cells, duct cells did not ac-
esize proteins. This study points
ncreatic duct cells are metabol-
ically active and that fasting was associated with reductions in glucose oxidation and alkaline phosphatase activity. Whether these metabolic activities are under the control of gastrointestinal hormones remains to be determined. Moreover, the significance of the reduction in alkaline phosphatase activity with fasting is not known.

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nce of C3H/HeJ Mice to Lethal Challenge with Herpes Simplex Virus (39983)

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luction. Herpes simplex virus (HSV) es *in vitro* in spleen cells of certain of mice after prestimulation with ıic doses of endotoxic lipopolysac- (LPS) (1). However, no viral repli- ɔccurs in unstimulated cultures or in ; prestimulated with T-cell mitogens, ing that B lymphocytes or macro- activated by LPS are the target cells V replication in mouse spleen cell ;.

der to provide more definitive data role of lymphoreticular cells in HSV ion *in vitro* we recently employed xin-unresponsive mice (C3H/HeJ) in eriments. This mouse strain is resist- number of toxic and immunological of LPS (2-5). Furthermore, both mphocytes and macrophages of this ıre unresponsive to LPS (6, 7). As ıted, spleen cells from C3H/HeJ etreated with LPS failed to prolifer- did not support virus replication *in* the same manner as did spleen cells e closely related C3HeB/FeJ mouse ; susceptible to the biological effects We therefore compared the suscep- of these two strains of mice to *in* ection with HSV.

rials and methods. Virus. HSV-1 strain was adapted to mouse brain d for *in vivo* experimentation (8). A infected brains was prepared, ho- zed, diluted 1/10 in saline, and in aliquots at −70°. This pool, con- 5×10^6 plaque-forming units (PFU)/ s used for all experiments. Before llular material was removed by cen- trifugation. For *in vitro* experiments, a tissue culture-derived preparation of HSV (WAL) grown in VERO cells and containing 5×10^8 PFU/ml was used. Viral titrations were performed in mouse embryo fibroblasts (MEF) which were derived from one batch of frozen cells and used between the third and ninth passage. The detail of the viral plaque assay have been described (1).

Mice. Male C3HeB/FeJ and C3H/HeJ mice, 8–16 weeks old, obtained from Jackson Laboratories (Bar Harbour, Maine) were injected with 10-fold serial dilutions of HSV in 0.1-ml (intraperitoneally, ip) or in 0.03-ml (intracerebrally, ic) aliquots. Deaths were recorded daily until 18 days after infection and the LD_{50} was calculated as described (8).

Spleen cell cultures. Single spleen cell suspensions were prepared using sterile technique and stimulated by mitogens in a standard lymphocyte stimulation assay (1). The following mitogens were used: PHA (purified phytohemagglutinin; Wellcome Laboratories, Bechenham, England, 2.5 μg/ml), Con A (concanavalin A; Calbiochem, San Diego, Calif., 2.5 μg/ml), and LPS (lipopolysaccharide W *Salmonella typhimurium* 3125-25; Difco, Detroit, Mich., 10 μg/ml). A crude preparation of *Acholeplasma laidlawaii* (AL) was prepared and used as a mitogen (submitted for publication).

For demonstration of viral replication in mitogen-stimulated spleen cell cultures a previously described technique was followed (1). After prestimulation with optimally stimulatory doses of mitogens for 2 days, cultures were infected with HSV-1 at a multiplicity of infection of 10. After 24 hr, the cultures were terminated by rapid freezing at −70°. Viral titers were determined by plaque assay in MEF (see above).

Peritoneal exudate cells. Mice were in-

work was initiated when S.E.M. was a Visitssor at the University of Heidelberg. Send reuests to Dr. Stephan E. Mergenhagen, Natitute of Dental Research, National Institutes ı, Bethesda, Maryland 20014.

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jected ip wth 10^5 PFU of HSV and sacrificed after 24 hr. Five milliliters of saline was injected ip and the cells of the peritoneal cavity (PEC) were recovered by standard techniques. The PEC were centrifuged, resuspended in medium, and directly plated on confluent monolayers of MEF for evaluation by the viral plaque assay.

Results. In vitro mitogenicity of LPS for spleen cells of C3H/HeJ and C3HeB/FeJ mice and replication of HSV in these cultures. As previously described by several laboratories (3, 5) spleen cells of C3H/HeJ mice did not respond to the mitogenic effect of LPS in culture, in contrast to spleen cells from C3HeB/FeJ mice (Table I). However, they responded well to AL, another B cell mitogen (unpublished observation) and to the T-cell mitogens PHA and Con A. The ability of C3H/HeJ mice to respond to B-cell mitogens other than LPS is well known (3).

We have previously reported that replication of HSV in mouse spleen cultures required lymphoid cell activation with a B-cell mitogen such as LPS (1). We therefore investigated whether HSV would replicate *in vitro* in C3H/HeJ spleen cells. There was no demonstrable HSV replication in LPS-treated C3H/HeJ cells ($<10^1$ PFU/ml), while HSV replicated very well in LPS-treated C3HeB/FeJ spleen cells (4.2×10^3 PFU/ml) (Table II). However, in spleen cell cultures of C3H/HeJ mice stimulated with the B-cell mitogen, AL, virus replication did occur (5.3×10^3 PFU/ml), thus demonstrating that C3H/HeJ spleen cells were capable of replicating HSV *in vitro* when appropriately stimulated.

TABLE I. EFFECT OF DIFFERENT MITOGENS ON SPLEEN CELL CULTURES OF C3H/HeJ AND C3HeB/FeJ MICE

	Lymphocyte proliferative response (cpm [^{3}H]TdR incorporated/6 × 10^5 cells)	
Mitogen	C3H/HeJ	C3HeB/FeJ
None added	1,733	1,991
PHA	63,774	74,954
Con A	101,301	111,419
LPS	1,946	45,745
AL	25,613	23,420

TABLE II. *In vitro* REPLICATION OF HSV IN SPLEEN CELL CULTURES OF C3HeB/FeJ AND C3H/HeJ MICE.

	HSV replication[a] (PFU/3 × 10^6 cells)	
Mitogen	C3H/HeJ	C3HeB/FeJ
None added	$<10^1$	$<10^1$
PHA	$<10^1$	$<10^1$
LPS	$<10^1$	4.2×10^3
AL	5.3×10^3	1.2×10^3

[a] Cultures were preincubated with or without mitogen for 48 hr, infected with HSV, and incubated for another 24 hr. Titers were determined in MEF in a viral plaque assay.

Lethality of C3H/HeJ and C3HeB/FeJ mice after ip and ic infection with HSV. We next determined the intrinsic susceptibility of these mice to ip challenge with HSV in the absence of any exogenously administered LPS. Quite surprisingly, we found that approximately 100 times more HSV was required to kill C3H/HeJ mice after ip inoculation than to kill C3HeB/FeJ mice (Table III).

It is thought that after ip inoculation, HSV first replicates in the peritoneum, then travels via the blood to the brain where it produces a fatal encephalitis (9). In order to determine if C3H/HeJ mice were resistant to HSV because of a failure of the virus to replicate in the cells of the target organ (brain), HSV was inoculated directly ic into both mouse strains. After ic inoculation, there was no significant difference in susceptibility between the two strains, although the LD_{50} was lower in both (Table III).

These findings suggested that the resistance of C3H/HeJ mice was due to a failure of HSV to replicate intraperitoneally. In order to determine if this was the case, 5×10^5 PFU of HSV were inoculated ip into both strains and virus titers were quantitated in their peritoneal cells 24 hr later. Viral recovery was more consistent and considerably higher from PEC of C3HeB/FeJ mice than from PEC of C3H/HeJ mice (Table IV). In four of six C3H/HeJ mice, no HSV was recovered from their PEC, and the remaining two mice yielded 5 and 17 PFU of HSV/ml, respectively. In contrast, six of six C3HeB/FeJ PEC contained HSV ranging in titer from 30 to 200 PFU/

LE III. LETHALITY OF C3H/HeJ AND C3HeB/FeJ MICE AFTER IP AND IC INFECTION WITH HSV.

t. No.	Type of infection	LD_{50} of HSV (PFU/mouse) C3H/HeJ	C3HeB/FeJ	P^a
I	ip	7.95×10^5	6.30×10^3	<0.05
II	ip	1.72×10^5	2.35×10^3	<0.05
II	ip	1.35×10^5	2.45×10^3	<0.05
V	ic	1.78×10^2	4.27×10^2	Not significant
V	ic	4.27×10^1	3.20×10^2	Not significant

stical evaluation performed using Student's *t* test.

IV. TITERS OF HSV IN PEC OF C3H/HeJ HeB/FeJ MICE 24 hr AFTER INFECTION OF 5 × 10^5 PFU OF HSV.

). Mouse No.	Recovery of HSV from peritoneal cells (PFU/ml)[a] C3H/HeJ	C3HeB/FeJ
1	0.5×10^1	8×10^1
2	0	1×10^2
3	0	7×10^1
1	1.7×10^1	2×10^2
2	0	9×10^1
3	0	3×10^1

were recovered from the peritoneal cavity, y washed, suspended in medium, and tested ent monolayers of MEF in a viral plaque

ıs, these findings suggest that the ce of C3H/HeJ mice to HSV is re- a failure of the virus to replicate itoneally in this strain.

ssion. We have recently described a of *in vitro* replication of HSV in pleen cell cultures prestimulated by . Unstimulated spleen cell cultures tures stimulated by the T-cell mito- IA and Con A did not support viral Since spleen cells of C3H/HeJ mice en shown to be unreactive to the of LPS (3, 5), it is not surprising lication of HSV could not be dem- d in these cultures. This observation o compare the lethality of HSV in ains of mice. In this report, we have that the mortality of C3HeB/FeJ er ip infection with HSV was signif- ıigher than that of C3H/HeJ mice. ference appears to be noteworthy) our knowledge, it represents the ıonstration of a difference in suscep- o a viral infection in these closely ıtrains of mice that were thought to differ only in their endotoxin responsiveness.

Host susceptibility to viral infections is obviously determined by a complex series of factors (10). In a recent study, Lopez suggested that resistance to HSV-1 in mice was determined by at least three genes (11). The C3H/HeJ mouse strain has been extensively studied since it is unresponsive to all known effects of endotoxin due to a mutation in a single autosomal gene that occurred some time between 1960 and 1965 (12, 13). Furthermore, this defect is highly specific, since it seems to be limited to the lipid A moiety of LPS (14). The C3H/HeJ and C3HeB/FeJ strains are closely related, and the only identified difference between them is their LPS responsiveness. Therefore, the fact that the C3H/HeJ strain is resistant to lethal infection with HSV-1 is extremely interesting since it raises the possibility that the gene that controls LPS sensitivity is also one of the genes responsible for susceptibility to HSV-1 infection.

Our evidence suggests that the resistance of C3H/HeJ mice to HSV is due to events taking place in the peritoneal cavity since HSV grows poorly in peritoneal cells of C3H/HeJ mice (Table IV), and since they appear to grow well intracerebrally in this strain (Table III). Furthermore, our evidence also suggests that resistance is due to some defect in the activation of cells, since spleen cells of this strain will replicate HSV normally *in vitro* when appropriately stimulated (Table II). Finally, that the connection between LPS and HSV sensitivity is significant and not merely coincidental is suggested by the findings that LPS is required for the replication of HSV *in vitro*, and that a mouse strain whose only known defective response is to LPS is also resistant to HSV.

While replication of HSV-1 within peritoneal cells appears to be one of the factors necessary for the induction of lethality, it is obviously not the only factor involved. HSV-1 replicates normally in (C57/BL/6 × DBA/2J) F1 mice even though these mice are resistant to its lethal effects (1). These findings are best explained by the hypothesis that more than one gene controls susceptibility to HSV-1 induced lethality (11).

It is not yet known whether HSV-1 replicates within the B lymphocytes or the macrophages in the peritoneal cavity. Since the responses of both cell types to LPS are abnormal in C3H/HeJ mice (7), either one or both cells could be the target. However, our previous findings suggest that the activated B cell is more important than the macrophage in this regard since HSV-1 replicates well in macrophage-depleted B lymphocytes but replicates only poorly in isolated macrophage populations (1).

It is premature to speculate on the relationship between the sensitivity of the mouse to HSV and LPS. First it must be demonstrated if this connection is real. A formal genetic analysis to ascertain whether the LPS gene controls HSV sensitivity is currently underway. However, if this does turn out to be the case, it would suggest that either LPS or some LPS-like molecule plays an important but heretofore unrecognized role in the homeostasis of the lymphoid system, or that the LPS gene regulates some important, non-LPS-related functions in the host. The implications of either possibility are potentially great.

Summary. In vitro replication of herpes simplex virus (HSV) in murine spleen cells requires simultaneous cell stimulation with a B-cell mitogen such as lipopolysaccharide (LPS). As expected, spleen cells of LPS-unresponsive C3H/HeJ mice did not support HSV replication in LPS-pretreated cultures, while spleen cells from closely related but LPS-responsive C3HeB/FeJ did. More importantly, the C3H/HeJ strain was found to be intrinsically resistant to HSV ir *in vivo*. After intraperitoneal (ip) i tion, HSV was 50–120 times mor lent for C3HeB/FeJ mice than for th HeJ strain. This resistance appeare due to a failure of HSV to repl C3H/HeJ peritoneal cells, since i infection with HSV, recovery of vi higher and more consistent from pe exudate cells of C3HeB/FeJ mice th C3H/HeJ mice. In addition, no dif in lethality was observed between th strains after a direct intracerebral i tion of HSV. This observation tha unresponsive mice are intrinsically r to lethal HSV infection, coupled v LPS requirement for HSV replica *vitro*, suggests an important but as ye plained link between host sensitivity and to LPS.

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rophotometric Assay of Catalase with Perborate as Substrate[1] (39984)

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udies on intracellular localization s, as determined by density-gra- uding zonal) centrifugation, it is o assay a number of enzymes on 40 samples. For many enzymes, used spectrophotometric assays pectrophotometer coupled to a (1). For catalase, however, al- ectrophotometric assays have ibed that use hydrogen peroxide e [for review, see (2)], they are le for assays of numerous tissue at by their nature are highly im- blems such as instability of the latency of particle-associated cat- high blank values made this ap- atisfactory for our purposes.

te was introduced as a substrate e by Feinstein (3). We used a ic adaptation of this procedure ral studies on catalase distribution utions of perborate are relatively d the problems of latency are hen the assay is carried out at erature or higher (7). However, tric and colorimetric methods are ming, of doubtful accuracy for ith low activity, and not readily to our data-processing system.

ort describes the use of perborate ate in a spectrophotometric assay in mouse liver fractions.

. The reaction mixture, under the finally established, consisted of 0.05 *M* potassium phosphate 7.4, and 0.002 to 0.05 ml of a ple diluted to effect an optical ange of 0.10–0.25/min. After on for about 5 min at 30°, the as started by the addition of 0.2 *N* sodium perborate, neutralized to pH 7.4 with 85% H_3PO_4 (4). Blanks without perborate were prepared for each sample.

Assays were carried out at 28–30° in quartz cuvettes specially cleaned to minimize the adhesion of bubbles to the surface of the glass (8). Spectrophotometric measurements were made at 220 nm, at intervals of 20–25 sec for a period of 2–3 min, with the apparatus described elsewhere (1).

To test the efficacy of the method, 3 ml of a mouse liver homogenate (1:10 in 0.25 *M* sucrose) was layered over a 42-ml sucrose gradient (0.29–0.88 *M*) containing 0.02 *M* ethanol in a 50-ml polycarbonate centrifuge tube. The tube was centrifuged at 4100 rpm for 70 min; 15 fractions of 3 ml each were collected and assayed. Details of gradient preparation and fraction collection have been previously presented (9). Catalase activity, as indicated by the first-order kinetic constant, was calculated by computing the least-squares fit of a plot of the natural logarithm of the optical density vs time. The slopes of these lines were converted to *k* values in terms of the total sample volume of 3 ml.

Results. Wavelength. Although the maximum absorption of perborate is at 205 nm, the contribution of the buffer and of tissue components is objectionably high at this wavelength. We have found that 220 nm represents the best compromise between sensitivity and blank absorption.

Buffer concentration and pH. Increasing the final concentration of phosphate from 0.047 to 0.093 *M* did not appreciably decrease the rate. However, higher concentrations (0.14 *M* and above) were inhibitory. The pH optimum was 7.40. A 10% decrease in reaction rate was observed when tris-(hydroxymethyl)-methylamine was substituted for phosphate.

Temperature. It has been most convenient to carry out the assays at 30°. At higher temperatures, the initial rates are faster, but as a result of enzyme inactivation the

pported by the U.S. Energy Research ment Administration. By acceptance of he publisher or recipient acknowledges ernment's right to retain a nonexclusive, icense in and to any copyright covering

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reaction obeys first-order kinetics for shorter periods of time.

Miscellaneous properties. Sucrose at final concentrations of 0.1 *M* does not affect the reaction; however, KCl is inhibitory at final concentrations above 0.02 *M*. Triton X-100 cannot be used in this assay because of its high uv absorption. However, digitonin (0.01%) and sodium cholate (0.25%) can be used without interference in tests for activation.

Applications. Figure 1 shows the results obtained with the original homogenate and with various fractions of the gradient. It is apparent that (a) the reaction obeys first-order kinetics over the assay period, (b) in each case the lines obtained by least-squares fit extrapolate to the same intercept, and (c) the slopes of the lines for a given sample are proportional to the volume of the sample used; the actual slopes for H_1 (0.01 ml) and H_2 (0.02 ml) were -6.80 and -13.24×10^{-4} sec^{-1}, respectively.

Table I lists the slopes (with standard errors) and intercepts for assays on the various fractions, calculated on the basis of the total fraction volume of 3 ml. The high values for fractions 1 and 2 reflect the soluble catalase from blood entrapped in the liver. Fraction 1 is the original homogenate layer, and fraction 15 is the pellet. The recovery in fractions 1 through 15 is 108.4% of the original homogenate. The average value of the intercepts is 0.2518 ± 0.0174 (1.286 ± 0.022 optical density units).

In a similar experiment, each fraction was assayed for catalase activity by both the spectrophotometric method described above and the colorimetric procedure (4). Regression analysis of the percentage of activity in each fraction as determined by both methods indicated a correlation coefficient of 0.96. The parameters of the distribution curves calculated from the percentages (9) were virtually identical: The midpoint values were 0.487 μm for the spectrophotometric assay and 0.498 μm for the colorimetric, and the corresponding values for the dispersions (σ) were 0.095 and 0.113 μm. These data are evidence that the two procedures are in fact measuring the same phenomenon, disappearance of perborate.

Discussion. The use of perborate as a substrate for the spectrophotometric assay of catalase seems entirely feasible, and superior in many respects to the use of hydrogen peroxide. The principal advantage is the stability of perborate solutions, which obviates the need for repeated standardiza-

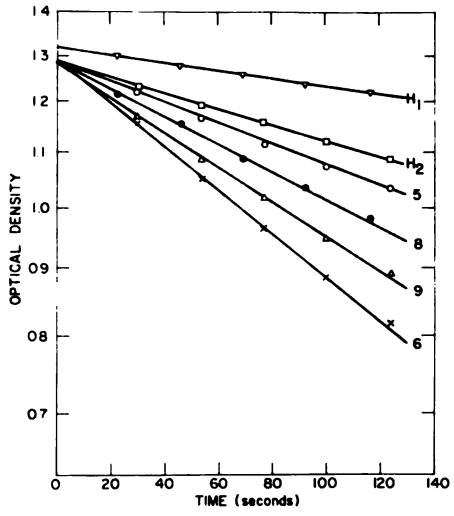

FIG. 1. Catalase assays on a mouse liver homogenate and on four fractions recovered after density-gradient centrifugation. The homogenate was diluted 1:30 in 0.05 *M*, pH 7.0, phosphate buffer; then assayed with 0.01 ml (H_1) and 0.02 ml (H_2) of the dilutions. The fractions were assayed with 0.01 ml (5), 0.02 ml (6 and 8), or 0.04 ml (9).

TABLE I. PARAMETERS OF CATALASE ASSAYS IN MOUSE LIVER HOMOGENATE AND FRACTIONS.

Fraction	d_m[a]	k ± SE (slope/3 ml)	Intercept
1	–	−0.8586 ± 0.0422	0.2677
2	0.100	−0.5297 ± 0.0202	0.2687
3	0.242	−0.3944 ± 0.0145	0.2760
4	0.316	−0.4460 ± 0.0103	0.2622
5	0.377	−0.5188 ± 0.0133	0.2618
6	0.430	−0.5250 ± 0.0188	0.2582
7	0.478	−0.3857 ± 0.0122	0.2640
8	0.525	−0.3441 ± 0.0054	0.2353
9	0.569	−0.2214 ± 0.0067	0.2592
10	0.612	−0.1298 ± 0.0049	0.2232
11	0.655	−0.0800 ± 0.0033	0.2549
12	0.698	−0.0423 ± 0.0020	0.2206
13	0.741	−0.0364 ± 0.0022	0.2294
14	0.786	−0.0308 ± 0.0011	0.2344
15	–	−2.0053 ± 0.0979	0.2524
Total		−6.5485	
Homogenate		−6.0390 ± 0.0929	0.2611

[a] Mean diameter (micrometers) of particulates in the fraction. See Ref. (9) for calculations.

and for correction for spontaneous de-
ıposition.
he spectrophotometric assay is also su-
or to the titrimetric and colorimetric
hods in which measurements are made
: single time point, and in which zero-
:r kinetics is generally assumed. We are
g the method described here routinely
:udies on rodent liver peroxisomes.
ummary. A spectrophotometric assay
catalase has been developed in which
um perborate, rather than hydrogen
›xide, is used as the substrate. The
hod is convenient, rapid, and readily
›ted to the measurement of catalase in
:ellular fractions.

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Receptors in the Canine Lung Which Respond to Vascular Pressure Changes (39985)

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Vagal afferent nerve activity from the lungs is involved in the dyspnea of heart failure. Constantine (1) found that pulmonary congestion increased the discharge frequency of slowly adapting stretch receptors. In addition, vagal afferent nerve activity from receptors located in the main branches of the pulmonary artery (2), pulmonary veins (3), and walls of intrapulmonary vessels (4, 5) which respond to vascular pressure changes were also reported. The functional relationships between vascular pressure and neural discharge frequency from intrapulmonary mechanoreceptors, however, have not been determined. The objective of the present study, therefore, was to determine the relationships between neural discharge frequency and pulmonary vascular pressures of receptors located within the lung.

Materials and methods. Afferent nerve activity was studied in four single and six small multifiber nerve preparations in five mongrel dogs (18–28 kg) anesthetized with 30 mg/kg of sodium pentobarbital (Nembutal, Abbott Laboratories). Ventilation and pulmonary arterial perfusion of the left lower lobe (LLL) were isolated from that of the animal, *in situ*, to permit optimum control of pulmonary hemodynamics and airway pressure. The LLL was exposed by removing ribs 2 through 8 on the left side. Left middle and upper lobes were ligated and removed. The airways of the LLL and right lung were separated by using a tube-within-a-tube system which allowed cannulation of the left bronchus.

Separate ventilation of the right lung and LLL was accomplished by a square-flow, solinoid respirator which enabled measurement of LLL static compliance and resistance as previously described (6). There were no appreciable differences in these measurements between the autoperfused LLL and the pump-perfused LLL during experimental manipulations. Systemic and LLL blood-gas tensions were maintained at a P_{O_2} of 100–300 mm Hg and a P_{CO_2} of 34–44 mm Hg at a pH of 7.35–7.44.

Two methods, described below, were used to systematically alter pulmonary vascular pressures.

Ramp changes in lobar vascular pressures (Method I). The LLL pulmonary artery and femoral vein were cannulated. Blood from the femoral vein was pumped through the lobe using a Sarns nonocclusive roller pump and returned to the animal via the intact lobar vein. Ramp changes in LLL vascular pressures were produced by either increasing pump speed or by tightening a snare placed around the lobar vein to increase outflow resistance.

Sinusoidal and step changes in LLL vascular pressures (Method II). Figure 1 shows the method used to produce sinusoidal and step alterations in LLL vascular pressures. The LLL vein, LLL branch of the pulmonary artery, and femoral vein were cannulated. Blood from a Mariotte bottle reservoir placed in a water bath (36–38°) was used to perfuse the LLL. Outflow blood was collected in a reservoir which could be raised or lowered to produce step changes in vascular pressures. Retrograde (vein to artery) and forward (artery to vein) perfusion of the LLL were used to produce differential alterations of venous and arterial pressures. Sinusoidal pulsations of desired amplitude and frequency were superimposed on a mean vascular pressure by using a Harvard respirator (Fig. 1). Sinuso-

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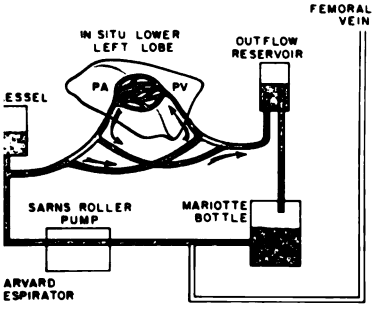


Schematic diagram of the *in situ* LLL per- :m. The stippled tubes show the retrograde ircuit. Arrows show the direction of blood oidal oscillations of vascular pressure were by changing the air pressure within the using a Harvard respirator.

ipulations enabled evaluation of discharge pattern over a wide vascular pressures and determina- ıe relative intrapulmonary location or venous) of the receptor (see

Statham pressure transducers d to monitor LLL airway and vas- :ssures. Manipulations of pulmo- cular pressures were performed LLL was apneic.

recording. Nerve activity was ob- om slips of nerve dissected from 'agosympathetic trunk and placed ngsten carbide bipolar electrodes l in a chamber filled with warmed)il. Nerve fibers selected for study)se responding to LLL vascular changes. Both single and small r nerve activity responded to vas- :ssure alterations. Nerve activity lified, filtered, and then, together . arterial, venous, and airway pres- corded on magnetic tape and dis- ı a Honeywell 1912 Visicorder.

'nalysis. The tape-recorded nerve 'as fed into a window discriminator ected action potentials on the basis tude. An upper voltage threshold cted to exclude action potentials desired amplitude, and a lower ıreshold excluded action potentials desired spike amplitude. A stan- put pulse was generated for each ike falling between these voltage ls. Thus, with small multifiber eparations a single active nerve fiber could be selected for analysis.

For sinusoidal variations of vascular pressures, the discriminator output was accumulated in 50-msec time intervals and averaged for eight pressure cycles using an Ortec Model 4620 histogram computer. The upstroke of the sinusoidal waveform was used to initiate the analysis of nerve activity, and LLL arterial and venous pressures. The resulting poststimulus time histograms and ensemble averages of LLL vascular pressures were plotted using a Honeywell 530 X-Y plotter (Fig. 3). The relationships between ramp changes of pulmonary vascular pressures and nerve activity were analyzed in a similar fashion, but the Ortec analyzer was triggered to begin the analysis manually.

Results. Ramp changes (Method I) of pulmonary vascular pressures were performed in five nerve preparations in four animals. Three fibers showed an increase in frequency of discharge when LLL venous pressure was increased by lobar vein constriction but not when pump speed was increased. Increasing pump speed increased LLL arterial pressure, but only small changes in LLL venous pressure occurred. One receptor was located within the lung close to the hilum by gently stroking the surface of the lung with an insulated probe. Inflating the lungs increased nerve discharge frequency. These receptors, therefore, were probably Hering–Breuer stretch receptors. The slopes of the linear regression lines (sensitivity) for nerve discharge frequency on venous pressure were 1.15 (F = 7.63, N = 34), 1.72 (F = 126, N = 168), and 0.510 impulses (imp)/sec/mm Hg (F = 57, N = 51) for these three nerve preparations. All regressions were significant ($P \leq 0.05$) indicating that impulse frequency was linearly related to pulmonary venous pressure. Venous pressure ranged from 0 to 40 mm Hg and impulse frequency from 0 to 90 imp/sec.

In two additional nerve preparations impulse frequency increased both when LLL vein was constricted and when pump speed was increased. These receptors did not significantly respond to inflation of the LLL. No adaptation of receptor discharge occurred when vascular pressures were held

constant for 1 min. The slopes of the regression lines of nerve activity on arterial pressure when pressure was increased by venous constriction were 2.98 (F = 188, N = 111) and 1.9 imp/sec/mm Hg (F = 155, N = 47); the slopes of the regression lines calculated when arterial pressure was increased by increasing perfusion pump speed were 1.45 (F = 92, N = 61) and 0.59 imp/sec/mmHg (F = 32, N = 45), respectively. The location of these receptors as either arterial or venous could not be firmly established using this method of perfusion.

Sinusoidal variations of LLL vascular pressures were used to define the stimulus-response characteristics and location of three pulmonary receptors in three animals. A representative response of nerve activity during pulsatile perfusion is shown in Fig. 2. Two frequencies of sinusoidal oscillation are depicted. Note that no significant change in airway pressure occurred. Poststimulus time histograms of data from this same nerve preparation are depicted in Fig. 3. It should be noted that as the pressure wave frequency increased, the venous pressure oscillation lagged the arterial. Examination, particularly of the highest frequency (56 cpm), indicates that cyclic nerve activity

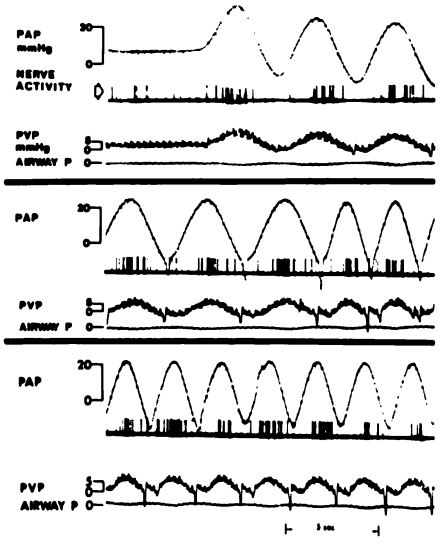


FIG. 2. Representative changes in nerve activity resulting from pulmonary vascular sinusoidal pulsation. The top trace is pulmonary arterial pressure (PAP); second from the top, nerve activity; third from the top, pulmonary venous pressure (PVP); fourth from the top, airway pressure.

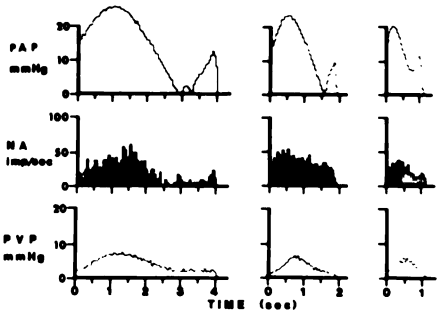
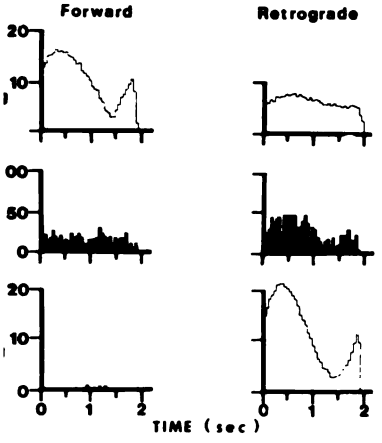


FIG. 3. Poststimulus time histograms of nerve activity and lobar vascular pressures at three pressure wave frequencies. The LLL was apneic and perfused in the forward direction. Top traces are histograms of pulmonary arterial pressure (PAP); middle, nerve activity (NA); and bottom, pulmonary venous pressure (PVP).

was more closely related to the arterial pressure wave than the venous pressure wave. This was confirmed by comparing correlation coefficients calculated for each pressure wave frequency studied. Correlations between LLL arterial pressure and nerve activity were r = 0.85 (N = 76) for 16 cpm, r = 0.80 (N = 38) for 42 cpm, and r = 0.84 (N = 22) for 56 cpm (all correlations were significant, $P \leq 0.05$). Correlations between venous pressure and nerve activity were r = 0.85 for 16 cpm, r = 0.49 for 42 cpm, and r = 0.43 for 56 cpm. The correlation between arterial pressure and nerve activity remained essentially the same as the pressure wave frequency increased but, the correlation between venous pressure and nerve activity progressively decreased. This receptor, therefore, was probably located in or near an intrapulmonary arterial vessel. Another receptor had similar response characteristics. Both did not respond to inflations of the LLL and slowly adapted to a constant elevated arterial pressure. There was a significant linear regression at the pressure wave frequencies studied with average slopes of 0.24 (F = 3.8, N = 136) and 2.44 imp/sec/mm Hg (F = 126, N = 161) for these two nerve preparations. Pulmonary arterial pressure ranged from 0 to 45 mm Hg, and impulse frequency ranged from 0 to 48 imp/sec.

Figure 4 is an example of poststimulus time histograms obtained from another



Representative poststimulus time histo-
obar arterial pressure (PAP), nerve activity
lobar venous pressure (PVP) during forward
erfusion (left) and retrograde pulsatile per-
ht). Records of nerve activity were from the
e preparation. The LLL was apneic during
ure manipulations.

eparation. Cyclic nerve activity oc-
/ith retrograde pulsatile perfusion
forward pulsatile perfusion. This
, therefore, was located in or near
ntrapulmonary venous vessel. Re-
ilar to those presented in Fig. 4
:ained at three additional pressure
quencies (i.e., 15, 23, and 34 cpm).
ere significant linear regressions (P
of nerve activity on venous pressure
:essure wave frequencies studied.
'age slope of these regression lines
imp/sec/mm Hg (F = 42, N =
his receptor did not significantly
to lung inflation.

sion. Three types of intrapulmo-
eptors have been extensively stud-
tant, Hering-Breuer stretch, and
monary capillary (type J) receptors
:he present study several Hering-
tretch receptors, identified by their
to lung inflation, responded to
ns in pulmonary venous pressure.
studies have shown that pulmonary
)n (1) and the cardiac pressure
. 8) can alter the discharge pattern
ıg-Breuer stretch receptors. The
hips between pulmonary vascular
and impulse frequency, however,
t determined. From results of the
tudy it appears that the relationship
between pulmonary venous pressure and impulse frequency was linear over a wide range of vascular pressure for those receptors responsive to pulmonary vascular pressure changes.

Five receptors which did not respond to inflation of the LLL but did respond to elevated pulmonary vascular pressure were also identified. Because these receptors did not show significant adaptation to constant elevated vascular pressures, it is unlikely that these receptors were irritant receptors (7). In addition, because there was an immediate increase in nerve activity upon elevation of lobar vascular pressure and the maximum impulse frequency of these receptors was greater than 20 imp/sec, it is unlikely that these receptors are type J receptors (7, 9). Three of these receptors were studied using sinusoidal oscillations of pulmonary vascular pressures. This method took advantage of the "low-pass filter" properties of the pulmonary vasculature (10). With this system it was possible to produce, at higher oscillation frequencies, arterial and venous pressure waves which were out of phase with one another. This permitted the approximate intrapulmonary localization (i.e., arterial side or venous side) of these receptors. Two of these receptors responded to arterial pressure changes.

Histologically, afferent nerve endings were located in the walls of the intrapulmonary arterial branches (11, 12) and venous vessels (11). In the present study receptors responding in a linear fashion to elevation of pulmonary arterial pressure were identified. A receptor which responded to venous pressure elevation was also found. It appears that receptors exist within the lung which may be similar to pulmonary arterial baroreceptors (2). These findings confirm several earlier studies (4, 5, 13) which identified intrapulmonary vascular receptors responsive to arterial and venous pressure pulsations. Further studies are needed to determine the role that these receptors may play during physiological and pathological alterations of pulmonary vascular pressures.

Summary. Intrapulmonary neural receptors responding to pulmonary vascular pressure changes were studied. Intrapulmonary receptors which could not be classified as

irritant, Hering–Breuer, or Type J were identified electrophysiologically. These receptors increased impulse frequency in a linear fashion with elevations of pulmonary vascular pressure. Receptors which responded to elevation of pulmonary arterial pressure and receptors responding to elevation of pulmonary venous pressure were identified. In addition, several Hering–Breuer stretch receptors which responded in a linear fashion to elevated pulmonary venous pressure were found. We conclude that elevation of pulmonary pressure can increase nerve impulse frequency from Hering–Breuer stretch receptors and from baroreceptors located in the intrapulmonary vasculature. Stimulation of these receptors may play a role in the reflex alterations of respiration during cardiac failure.

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:rythrocyte Glucose-6-Phosphate Dehydrogenase (G-6-PD) Deficiency on Light-Induced Riboflavin Deficiency in the Neonate[1] (39986)

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rapy has been adopted as a non-
atment for mild hyperbilirubine-
ıan newborns since it results in
rum bilirubin levels. We have
ed that this therapy may result
ı deficiency in the neonate with-
·6-phosphate dehydrogenase (G-
iency (1). Riboflavin deficiency
ined by measurement of eryth-
ıthione reductase (EGR) activ-
e adenine dinucleotide (FAD)-
enzyme. Subjects with G-6-PD
ıave significantly elevated EGR
cyte FAD levels (2–4). FAD is
to light than riboflavin (5). We
whether G-6-PD deficiency
ent the decrease in EGR activity
ith hyperbilirubinemia undergo-
erapy.

ınd methods. The study group
f five black neonates with G-6-
cy who developed moderate hy-
emia. Four of these infants were

ria for phototherapy were either
of jaundice associated with pre-
serum bilirubin above 10 mg/dl
ıl direct bilirubin) before 72 hr

erm infant was fed Similac for-
ve 20 calories/oz. Two of the
received Similac 60/40 at the
c concentration. The remaining
received intravenous fluids
the duration of this study (Nos.
Table I). None of the infants
tamin supplements during the
l.

tigation was supported (in part) by Gen-
Support Grant RR-05398 from the
arch Support Branch, Division of Re-
ces, National Institutes of Health.

The undressed infants with shielded eyes were in open cribs or isolettes with a bank of eight Westinghouse blue fluorescent lamps (F20T12B) of 20 W each, 56 cm from the skin surface. A Plexiglas shield over the bulbs protected them from ultraviolet irradiation. Lamps were changed every 200 hr.

Serum bilirubins were determined from venous blood by the method of Malloy and Evelyn (6).

The dye decolorization test of Motulsky and associates as described by Dacie and Lewis (7) was used for the initial screening for G-6-PD deficiency. Quantitative assays for erythrocyte G-6-PD were performed by the method of Zinkham (8). Values below 120 units/100 ml of RBC for female neonates and below 100 units/100 ml of RBC for male neonates indicate deficiency of this enzyme.

Erythrocyte glutathione reductase was determined by a modification of the method of Glatzle *et al.* which has been described in detail (9). The activity coefficient (AC) is a measure of the degree of saturation of the enzyme with the coenzyme. Normal AC values for neonates range from 0.9 to 1.2 (9).

The results of these studies were compared to those of the 21 infants with moderate hyperbilirubinemia and without G-6-PD deficiency who underwent continuous phototherapy described in detail previously (1). The two studies were run concurrently.

Results. The sex, birth weight, age at initiation of light therapy, duration of light therapy, quantitative G-6-PD values, and serum bilirubin and AC values before and after phototherapy are given in Table I.

The G-6-PD levels of the erythrocytes obtained prior to phototherapy ranged from 21 to 73 units/100 ml of erythrocytes, well

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TABLE I. DATA FOR NEONATES WITH G-6-PD DEFICIENCY BEFORE AND AFTER PHOTOTHERAPY.

Infant	Sex	Birth weight (g)	Age at light therapy (hr)	G-6-PD (units/100 ml of RBC)	Initial values		Duration of light therapy (hr)	Posttherapy values	
					Bilirubin (mg/dl)	AC		Bilirubin (mg/dl)	AC
1	F	2990	62	46	12.3	1.0	48	9.0	1.0
2	F	2410	65	21	14.5	–	90	7.5	0.95
3	M	1420	18	30	6.0	1.0	120	7.0	0.95
4	F	1900	30	28	9.5	0.9	72	5.5	0.95
5	M	1585	40	73	8.0	1.0	52	8.0	1.0

below the lower limits of normalcy for both males and females.

The initial and final AC values were all within the normal range of 0.9 to 1.2. In the case of infant No. 2 where no initial AC value was obtained, the value of 0.95 after phototherapy signified that no biochemical evidence of riboflavin deficiency occurred during the 90 hr of continuous exposure to light. The duration of phototherapy for the five infants ranged from 48 to 120 hr.

In our previous study of icteric neonates without G-6-PD deficiency, 15 of 16 infants undergoing phototherapy for 48 hr or more developed AC values of 1.3 to 2.0, indicative of riboflavin deficiency (1). The difference between the AC values in that study and those of the five neonates with G-6-PD deficiency reported here is highly significant ($P < 0.001$ by the Fisher exact test).

Discussion. The neonate is particularly susceptible to destruction of its blood riboflavin during phototherapy because of the relatively high levels of free riboflavin. It is this that creates the vulnerability since free riboflavin is considerably more sensitive to light than FAD (5). In the maternal plasma, FAD constitutes the major portion of total riboflavin. However, FAD does not pass across the placental barrier but is degraded by placental enzymes to free riboflavin which is secreted into the fetal blood. About 60% of total fetal plasma riboflavin is free riboflavin (10).

As previously shown, neonates with normal levels of erythrocyte G-6-PD subjected to phototherapy for periods over 48 hr developed riboflavin deficiency. The deficiency was measured by the decrease in saturation of EGR as determined by elevated AC values (1). The decrease in saturation of the apoenzyme with FAD coenzyme is a sensitive indicator of riboflavin deficiency in man (11).

That the EGR pathway is closely connected to the G-6-PD activity of the erythrocyte is shown in the following equations:

$$\text{glucose-6-PO}_4 + \text{NADP}^+ \xrightarrow{\text{G-6-PD}} \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+$$

$$\text{GSSG (oxidized glutathione)} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{EGR-FAD}} 2\text{GSH (reduced glutathione)} + \text{NADP}^+$$

Neonates with erythrocyte G-6-PD deficiency have a reduced ability to make NADPH. However, their EGR-FAD levels are elevated, apparently as a compensatory mechanism to produce reduced glutathione in the presence of low levels of NADPH (2, 3). It was postulated that erythrocytes of G-6-PD-deficient subjects have an enhanced uptake of the free riboflavin which is then converted to FAD (4). As a result, the blood of the G-6-PD-deficient neonate has a higher FAD content in the erythrocytes and lower free riboflavin in the plasma. This relationship and the greater stability of FAD to light may explain the absence of riboflavin deficiency in the five neonates with G-6-PD deficiency undergoing phototherapy in the study described in this paper.

Studies in this paper and those by Meloni *et al.* (12) have demonstrated that continuous phototherapy of G-6-PD-deficient infants up to 120 hr resulted in no increase in serum bilirubin levels. Where the line between beneficial and harmful effects of phototherapy is in these neonates remains to be determined.

Summary. Five neonates with moderate hyperbilirubinemia and G-6-PD deficiency underwent phototherapy for periods of 48 to 120 hr. No biochemical evidence of riboflavin deficiency resulted. This contrasts

vith evidence of riboflavin deficiency observed in 15 of 16 infants with moderate iyperbilirubinemia but no G-6-PD deficiency who were exposed to light for periods of 48 hr or longer. The elevated FAD levels of the erythrocyte of the G-6-PD-deficient nfants may have protected them from the ight-induced vitamin deficiency.

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Duration of the Fluoride-Induced Urinary Concentrating Defect in Rats (39987)

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Introduction. In addition to its role as the major fluoride excretory organ, the kidney appears to be one of the first organs to show changes in function following acute exposure to high doses of fluoride. This may not be due to any particular sensitivity on the part of the kidney (1) but, rather, to the locally high fluoride concentrations which are established during urine formation (2).

Many studies on the acute renal toxicity of fluoride have used the single, high-dose technique and the time course of toxicity has generally not been followed beyond a few hours (3, 4). There are, however, reports which suggest that acute fluoride exposure may produce prolonged renal dysfunction. Gottlieb and Grant (5) noted a strongly alkaline urine which persisted for 1 week after a single iv dose of fluoride. Taylor *et al.* (6) noted increased urine output and protein excretion and decreased urine specific gravity and sugar excretion for several days after iv administration of near-lethal doses of fluoride. Other studies (7, 8) have described a chronic renal concentrating defect which may, in rare cases, persist for months following methoxyflurane anesthesia. There is considerable evidence to suggest that the immediate concentrating defect seen after methoxyflurane anesthesia is due to elevated body fluid fluoride concentrations (2, 9–11) which are derived from the biotransformation of the anesthetic molecule (12). These elevated fluoride concentrations, as well as the renal concentrating ability, usually return to normal within a week. The chronic concentrating defect has not been linked to a residual fluoride effect but this possibility cannot be discounted.

The present report examines the relationships between declining body fluid fluoride concentrations and renal tissue solute concentrations, urinary osmolality, glomerular filtration rate, and the excretion rates of sodium, potassium, and chloride.

Methods. Experiment 1. Female, 170- to 200-g rats of the F-344 strain were anesthetized with sodium pentobarbital, 40 mg/kg, ip. Surgical preparation consisted of bilateral cut-downs over the femoral veins. One vein was used for the constant infusion of the sustaining infusion at 25 μl/min. The other vein was used for the hydroxymethyl [^{14}C]inulin prime injection (0.13 μCi in 0.20 ml of isotonic saline). Sustaining infusions consisted of 145 m*M* sodium and varying concentrations of chloride and fluoride. For the *F*(0)[1] (saline control) groups, the sustaining chloride concentration was 145 m*M;* for the *F*(125) group the sustaining chloride and fluoride concentrations were 140 and 5 m*M*, respectively; for the *F*(250) groups these concentrations were 135 and 10 m*M*, respectively. All infusates contained tracer hydroxymethyl [^{14}C]inulin. After starting the sustaining infusion and administering the [^{14}C]inulin prime dose, a 0.5-hr equilibration period was allowed. Three 0.5-hr clearance periods were taken and the infusions were then stopped. No other infusions were subsequently given to any group. Urine and plasma samples were collected as previously described (2).

Three *F*(0) and three *F*(250) rats were sacrificed at the end of the third 0.5-hr urine collection period. Following closure of the incisions, all other rats were returned to their cages. Three *F*(0) and three *F*(250) rats were brought to the laboratory 3 hr later. Two more 0.5-hr urine collections were made before sacrifice. At 22.5 hr after stopping the infusion, three *F*(0) and three *F*(250) rats were similarly studied, except that three 0.5-hr urine collections were taken. At 39 hr after stopping the infusion, four *F*(0), three *F*(125), and three *F*(250)

[1] The fluoride infusion rate is denoted by *F*(*x*), where *x* is the number of nanomoles of fluoride ion infused per minute.

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vere similarly studied. A single, termi-
lood sample was collected from each
ır plasma fluoride analysis. Upon sac-
, the kidneys were rapidly removed,
ıned into cortex and inner medulla,
ıprepared for analysis as previously de-
:d (2).
dium and potassium were determined
amephotometry, chloride by electro-
c titration, osmolality by freezing point
:ssion, and fluoride with the ion-spe-
:lectrode following HMDS diffusion as
ously described (2). Hydroxymethyl
nulin was determined by liquid scintil-
ı counting. All data are presented as
ıeans ± SEM. Statistical analyses for
ences between the means of the fluo-
nfused and control groups were done
Student's *t* test, two-tailed, unpaired.
ıignificance level was set at $P < 0.01$.
ɔeriment 2. The results of Experiment
gested the possibility that the glomer-
filtration rates of the *F*(125) and
)) groups continued to be depressed
after plasma fluoride concentrations
eturned to control levels. This experi-
was designed to test that possibility.
rats of the same strain, sex, and body
ıt were prepared for study as described
:periment 1. Three rats were assigned
ch of the fluoride infusion rate groups
, *F*(125), and *F*(250)]. The animals
primed with [^{14}C]inulin and infused as
ibed previously. Following three 0.5-
earance periods, all rats received a
ide-free isotonic saline infusion con-
ıg [^{14}C]inulin for 3.5 hr. During the
ıour of this infusion, plasma and urine
les were taken for two more clearance
minations. All rats were then returned
eir cages. Twenty-two hours later, the
were again anesthetized, primed with
inulin, and infused with isotonic saline
ıining [^{14}C]inulin at 25 μl/min. Follow-
0.5-hr equilibration period, three 0.5-
earance periods were completed. In
experiment, only the clearance of
inulin was studied.

sults. Experiment 1. Figure 1 presents
from the three 0.5-hr clearance periods
ı during the initial infusion. The top
ɔr each infusion rate group is the mean
for the first 0.5-hr period. The middle

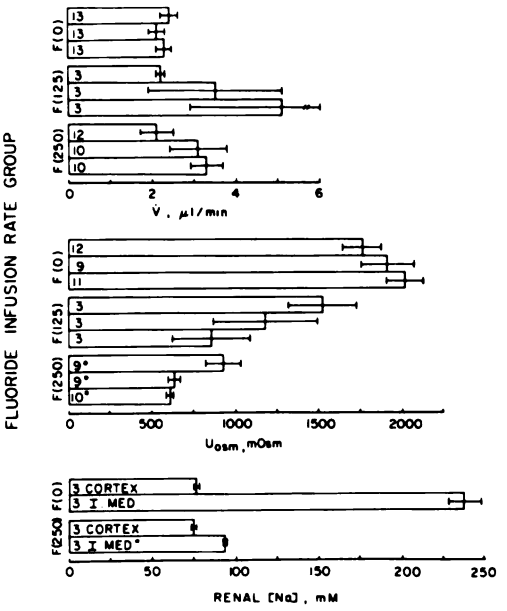

FIG. 1. Urine flow rates ($\dot{V}$), urine osmolalities, and renal tissue sodium concentrations during the fluoride infusion period of Experiment 1. Top, middle, and bottom bars are data (means ± SEM) from the first, second, and third clearance periods, respectively. Numbers in the bars indicate the observations. Asterisks indicate $P < 0.01$ compared to the *F*(0) group.

and bottom bars are for the second and third 0.5-hr periods, respectively. Each fluoride group showed a modest but not statistically significant increase in urine flow rate ($\dot{V}$) compared to the *F*(0) control group. Urine osmolalities for the *F*(0) group tended to increase slightly with time, while those of the fluoride groups showed dose-related decreases. The reductions were statistically significant in the *F*(250) group. At the end of the initial infusion period, the mean renal inner medullary sodium concentrations for the *F*(0) and *F*(250) groups were 238 ± 10 and 94 ± 1 m*M*, respectively. The corresponding renal cortical sodium concentrations showed no difference at 77 ± 2 and 75 ± 1 m*M*. Cortical and inner medullary chloride concentrations paralleled those of the sodium concentrations. These data indicate that the concentrating defect was established during the brief fluoride exposure.

Figure 2 shows the excretion rates of the major endogenous solutes, sodium, potassium, and chloride. The top bar for each

infusion rate group is the mean for the three 0.5-hr collections during the initial infusion. The second, third, and fourth bars for the $F(0)$ and $F(250)$ groups are the mean values observed for the collections taken at approximately 4, 24, and 40 hr after stopping the infusion, respectively. The bottom bar for the $F(125)$ group represents data obtained 40 h after stopping the initial infusion (there were no 4- or 24-hr collections for this group).

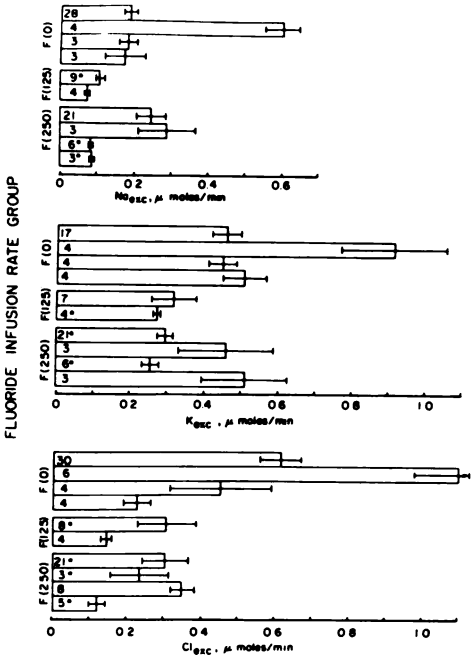

FIG. 2. Excretion rates of sodium, potassium, and chloride for each infusion rate group during the fluoride infusion period (top bars) and up to 40 hr (bottom bars) after stopping the fluoride infusion in Experiment 1. The second and third bars of the $F(0)$ and $F(250)$ groups represent data obtained at 4 and 24 hr after stopping the fluoride infusion, respectively. Means ± SEM; N given in bars. Asterisks indicate $P < 0.01$ compared to the $F(0)$ group.

The data in Fig. 2 show generally depressed solute excretion rates in the $F(125)$ and $F(250)$ groups for each observation period. This depression was greater for sodium and chloride than for potassium. The reductions in sodium and chloride excretion attained statistical significance for the $F(250)$ group even at 40 hr after stopping the initial infusion. Urine flow rates of the fluoride groups at 4, 24, and 40 hr were not statistically different from those of the saline-infused control group.

Glomerular filtration rates observed during the initial infusion are shown in Table 1. These rates were depressed in the fluoride groups in a dose-response manner.

Figure 3 shows the time course of inner medullary sodium concentrations, urinary osmolalities, and plasma fluoride concentrations. The large decrease in inner medullary sodium concentration of the $F(250)$ group present at the end of the initial infusion showed only a slight recovery 4 hr later even though the mean plasma fluoride concentration had fallen from 211 to 51 μM. Subsequently, there was a progressive return of inner medullary sodium concentra-

TABLE 1. EXPERIMENTS 1 AND 2. TIME COURSES OF GLOMERULAR FILTRATION RATES (MICROLITERS PER MINUTE).

Experiment 1				Experiment 2			
Clearance period	Fluoride infusion rate group			Hours after F^- infusion	Fluoride infusion rate group		
	$F(0)$[a]	$F(125)$	$F(250)$		$F(0)$	$F(125)$	$F(250)$
1	1282[b]	1124*	944*	0[c]	1196	1045	872*
	±46	±22	±55		±72	±99	±63
	13	3	11		9	9	8
2	1300	999*	780*	3	1157	930*	677*
	±77	±53	±104		±62	±35	±38
	12	3	12		6	6	6
3	1260	795*	645*	24	1243	919*	598*
	±77	±66	±62		±91	±58	±72
	13	3	11		9	8	9

[a] $F(x)$ = fluoride infusion rate in nanomoles per minute.
[b] Mean, SEM, N.
[c] During fluoride infusion.
* Significantly different from $F(0)$ group at $P < 0.01$.

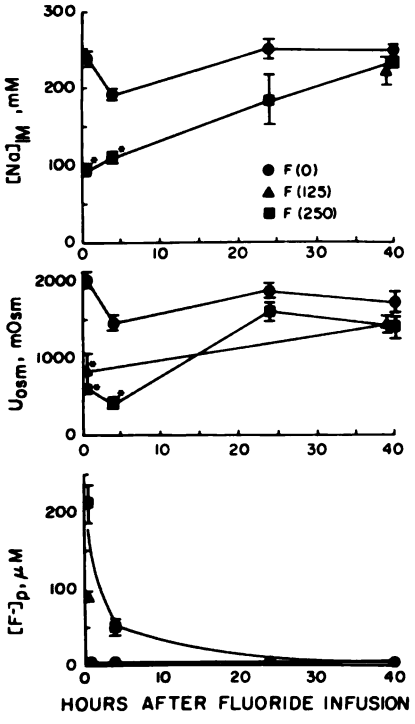

FIG. 3. Time course of renal inner medullary sodium concentration, urine osmolality, and plasma fluoride concentration relative to the period of fluoride infusion (0 time on the graph). Asterisks indicate $P < .01$ compared to the $F(0)$ group.

on to control levels which, at 40 hr after uoride exposure, remained slightly lower $P > 0.1$) than that of the control group. or the $F(125)$ group, inner medullary sodium concentration was determined only at) hr and this value was not significantly ifferent from that of the control group. ualitatively similar results were observed r the time course of urine osmolality. The lasma fluoride concentrations of the $F(0)$ roup were approximately 2 μM throughout ie experiment. All plasma, renal cortical nd inner medullary fluoride levels for the (250) group are shown in Table II. At 40 r for the $F(125)$ group, these values were .2 ± 0.5, 42 ± 3, and 127 ± 37 μM, spectively. Although renal tissue fluoride oncentrations of the $F(0)$ group were not etermined in this experiment, the 40-hr oncentrations for the fluoride groups were ithin the range found for stock rats which ad received no anesthesia or infusions (cor- x = 36 ± 9 μM, inner medulla = 94 ± 2 μM; unpublished data).

Experiment 2. Table I presents the glomerular filtration rate time course. During the fluoride infusion period, the values were similar to those observed in Experiment 1 (Table I). During the 3rd and 24th hr after stopping the fluoride infusion, glomerular filtration rates were further reduced in the $F(125)$ and $F(250)$ groups. Compared to the control group and at 24 hr after the infusion, the reductions were 26 and 52% for the $F(125)$ and $F(250)$ groups, respectively. The data given above and in Table II suggest that the renal tissue and plasma fluoride concentrations at this time were approximately the same in each group.

Discussion. Two previous studies have investigated the influence of acutely elevated body fluid fluoride concentrations on the immediate changes in the urinary concentrating abilities of rats (2) and dogs (13). These studies reported qualitatively similar results which included increased urine flow rates, either a decrease or no change in the excretion rates of sodium, potassium, and chloride, and decreases in urinary osmolality, glomerular filtration rate, free water reabsorption, and inner medullary sodium concentrations. The fluoride-induced reduction in inner medullary solute concentration was viewed as the most probable primary event to account for the other features of the concentrating defect (2). Both reports suggested increased vasa recta blood flow

TABLE II. EXPERIMENT 1. TIME COURSE OF PLASMA AND RENAL TISSUE FLUORIDE CONCENTRATIONS (MICROMOLAR) FOR THE $F(250)$ GROUP.

Hours after infusion	Plasma	Cortex	Medulla
0	211[a]	381	784
	±26	±14	±42
	10	3	3
4	51	152	525
	±10	±24	±180
	3	3	3
24	3.4	78	93
	±0.2	±24	–
	3	3	1
40	2.6	55	109
	±0.3	±8	±13
	3	3	3

[a] Mean, SEM, N. Renal tissue concentrations expressed in terms of tissue water.

and subsequent medullary solute washout as an underlying mechanism.

The current studies support the above findings and, further, demonstrate that the depression of renal function persists for many hours after plasma fluoride concentrations have returned to control levels. During the brief exposure to fluoride, urine flow rates were elevated, although not with statistical significance, and urinary osmolalities and inner medullary sodium concentrations were reduced (Fig. 1). These major indicators show that the acute fluoride-induced concentrating defect was established during the 2-hr fluoride infusion period. Further, glomerular filtration rates were reduced in a dose- and time-related manner (Table I), while the excretion rates of the major endogenous solutes were variably reduced (Fig. 2, top bars). The changes in solute excretion can, in large part, be accounted for by changes in glomerular filtration, as has been reported (2).

The persisting effects of acute fluoride exposure are shown in Figs. 2 and 3 and Table I. In spite of the rapidly declining plasma fluoride levels, the reduced inner medullary sodium (and chloride) concentrations and urinary osmolalities in the *F*(250) group showed essentially no change at 4 hr and the solute excretion rates were depressed relative to the *F*(0) group. At 24 and 40 hr after the fluoride infusion, the inner medullary sodium concentrations and urinary osmolalities of the fluoride groups remained lower than those of the *F*(0) group, although the differences were not statistically significant, and the solute excretion rates remained generally depressed.

The glomerular filtration rates of the *F*(125) and *F*(250) groups did not recover but, in fact, continued to decline for at least 24 hr after the fluoride infusion (Table I). The data in Table II indicate that these several persisting effects were not related to continuously elevated plasma or renal tissue fluoride concentrations.

The failure of inner medullary solute concentrations, solute excretion, and urinary osmolality to recover in proportion to declining plasma and renal tissue fluoride concentrations may be related to continuously depressed glomerular filtration rates. As discussed elsewhere (2), the generation of a hypertonic inner medullary interstitium is dependent on the delivery of an adequate solute load to the ascending limb of the loop of Henle, among other factors. However, the initial decrease in medullary solute concentration need not be related to decreased glomerular filtration rate (2).

The mechanism whereby fluoride depresses glomerular filtration rate remains unknown. A preliminary report (14) described up to 50% reductions in rat glomerular filtration rate, while simultaneously determined systemic arterial blood pressure, renal blood flow and para-aminohippuric acid extraction showed insignificant changes. The possibilities of a fluoride-induced chronic alteration in the permeability characteristics of glomeruli and/or the distribution of intrarenal blood flow must, therefore, be considered.

Summary. The changes in rat renal function produced by acute exposed to graded doses of fluoride were measured over 2 days. During the 2-hr iv infusion, urine flow rate increased moderately in the fluoride groups, while urinary osmolality, inner medullary sodium and chloride concentrations, glomerular filtration rate, and the excretion rates of sodium, chloride, and potassium decreased in a dose–response manner. Four hours after terminating the fluoride infusion, plasma fluoride concentrations had fallen 75% (peak value, 211 μM). This decline was not accompanied by recovery in either urinary osmolality or medullary sodium and chloride concentrations, while at 24 and 40 hr, these values showed a gradual return toward control values. Solute excretion rates, especially of sodium and chloride, were depressed even at 40 hr. Glomerular filtration rate continued to fall throughout the first day after stopping the fluoride infusion. These results suggest that the delayed recovery in urinary osmolality and medullary solute concentrations, as well as the continued depression in solute excretion, are related to the prolonged fluoride-induced decrease in glomerular filtration rate.

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Radiosensitivity of the Human Parotid Gland (39988)

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The severe xerostomia generally associated with cancerocidal levels of radiotherapy administered to head and neck malignancy patients makes oral health maintenance exceedingly difficult. Meticulous oral hygiene and vigorous fluoride therapy are essential in protecting the teeth from rampant dental caries (1-5) and a saliva substitute can be very helpful in overcoming the soft tissue problems that accompany the xerostomia (6).

These troublesome intraoral problems result from the depression of salivary gland function by the irradiation (7-11). We have previously reported (12) that the rate of unstimulated whole saliva flow decreases progressively during radiotherapy to the point that, after 6 weeks, only 5% of the original flow remains. It has been suggested that the parotid is more radiosensitive than the other salivary glands (13-16) and salivary gland extirpation experiments (17) have indicated that the level of caries developed is in direct proportion to the amount of serous glandular tissue removed. There is thus the indication that the rate of function of the parotid gland may be of critical importance in preserving the teeth of the irradiated patient.

The present study evaluates the resting parotid flow rate response in patients receiving radiotherapy involving the salivary glands.

Materials and methods. Patients were seven white males ranging in age from 42 to 71 years, with primary malignant lesions in the head and neck area. Lesions were located in either the base of the tongue, the soft palate, the hard palate, or the retromolar trigone. The fields were such that the parotid glands were totally within the beam. Prior to the initiation of radiotherapy a series of parotid saliva samples was lected to establish baseline values. Sam was undertaken at about 7:30 AM wit patients fasting. Patients were seated fortably in a semi-isolated area and e neous interferences were not allowed patient was instructed not to engaj conversation and to reduce oral excu to a minimum. Every effort was ma collect, as nearly as possible, the true re secretion of the parotid gland. A vac maintained sampling device (18) was tioned over the orifice of the parotid with an absolute minimum of manipul and the patient sat quietly over a peri 0.5 hr. Accumulating parotid saliva collected in a graduated tube and a ra flow was expressed in milliliters per mi The device was drained thoroughly each sampling to assure that any s within its confines would be harvested.

The first day's collection for each pa was considered a familiarization proce and no data were recorded. Three pre ment control samples were subsequ collected from each of five of the pa and two were obtained from each o remaining two patients. In each inst the last baseline sample was collecte the morning of and just prior to the treatment. Irradiation was administere parallel opposed fields from a cob source and was given at a daily dose of 225 rads, customarily on a Mo through Thursday schedule each week tempts to collect saliva continued thr out the first 4 weeks of therapy and rates were recorded for any period in v flow was noted.

Results and discussion. Resting flow responses are outlined in Table I. The treatment flow rate mean (19 samples seven subjects) was 0.045 ml/min (S 0.023). This is an acceptable baseline f since in previous work (19) with over healthy young adult males we found a

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TABLE I. RESTING PAROTID FLOW RATE RESPONSES TO RADIOTHERAPY.

Resting parotid flow rate (ml/min)				
Pretreatment			Number of treatments (225 rads)	
Number of samples	Mean	SD	1	2
3	0.057	0.006	0.030	0
3	0.073	0.028	0.040	0
3	0.040	0.013	0.010	.005
2	0.030	0.000	0.001	0
2	0.070	0.014	0.060	0
3	0.012	0.004	0.005	0
3	0.032	0.003	0.012	0

ati-

:an value of 0.040 ml/min (SD =

morning after the first radiother-
ıent (approximately 24 hr after
reatment) the parotid flow rate
decreased to 0.023 ml/min (SD
This virtually 50% reduction in
w resulted from the administra-
y 225 rads. At the next sampling
4 hr later (24 hr after radiother-
ent No. 2), there was no trace of
ı of the seven patients. In the
tient a total of 0.15 ml of saliva
ed in the 30-min period; rate of
hus only 0.005 ml/min for this
here was no quantifiable flow in
se patients after this time. On
he inner surface of the metal
ap might appear damp, but not
rop of saliva was present.

ough resting flow from the pa-
virtually ceased in these patients,
induce immediate complaints as-
ith intraoral dryness. Clinically,
appeared somewhat thickened
ut it was evident that extraparotid
tained the ability to maintain a
adequate degree of moisture in
. This supports previous work on
ɔution of the parotids to whole
ıme. Gore (20) found that the
rovided slightly more than 50%
le saliva output when exogenous
were not employed. Schneyer
(21) noted a 26% contribution
cts at a very low state of stimula-
a *et al.* (22) studied two subjects
that the parotids contributed
e resting saliva flow. In 71 sub-
ɔrs (23) found that the subman-
dibular glands contribute more than three times as much resting flow as do the parotids. Kerr (24) also found that, under resting conditions, the submandibular glands secrete at a rate about three times that of the parotids. Shannon (25) has reported data from 63 healthy young adult males indicating that the parotid glands contribute an average of 32.9% to the total resting salivary flow. In general, these observations support the clinical finding that extraparotid saliva sources are adequate to maintain a wet state in the mouth even when resting parotid flow has been obliterated.

The results of this study support the widely held concept that a marked depression in salivary flow results when the salivary glands are exposed to radiation. It provides additional information, however, in that the flow rate contribution of the parotid gland to whole saliva flow is almost completely eliminated after only two irradiation treatments of 225 rads each. Our previous work with whole saliva indicated a 60% loss in flow after 1 full week of treatment and steadily decreasing residual flow rates of 29, 24, 19, 9, and 5%, after 2, 3, 4, 5, and 6 weeks of treatment, respectively (12). It is apparent that most of the oral fluid being produced in postradiotherapy patients is derived from mucous glandular components and that the parotid contribution is negligible.

It has been known for many years that salivary gland changes transpire within a short time following irradiation. It was pointed out in 1911 (26) that swelling occurs within a few hours, and in 1912 Ceresole (27) observed that this swelling began

after about 5 hr and peaked at about 10 hr after treatment. Kashima *et al.* (28) studied surgical specimens from 22 patients that had been irradiated. Ten irradiated parotid glands were examined microscopically and all exhibited acute inflammatory and degenerative changes. The discrete outlines of the acini were barely discernible and the serous cells had large irregular basilar vacuoles that seemed to rupture cells and cleave them free from the basement membrane. Fourteen submandibular glands were similarly examined and the serous acini showed all of the degenerative changes described for the parotid glands. In sharp contrast, little if any discernible microscopic change was present in mucous cells. It was concluded that the primary damage from irradiation was in the serous cells, that this brings about the release of amylase to produce the characteristically observed hyperamylasemia, and that this degeneration brings about the marked inflammatory cell infiltration found in these glands. It was also noted that prompt and near-total cessation of salivary gland secretion occurred early in the postirradiation period. Silverman and Chierici (13) and Rubin and Doku (16) also stated that serous acini are more sensitive to irradiation than mucous-producing components, but no supporting data were provided.

Eneroth *et al.* (14) collected resting parotid saliva from the eight glands of four patients undergoing radiotherapy. The mean rate of flow decreased from 57 mg/min (SD = 85) to 12 mg/min (SD = 22) after administration of 550–700 rads. For a group of 11 parotid glands they found that the flow fell to less than 60 mg (1 drop) per 10-min collection period after a 550- to 700-rad dose. Four other glands required 1000–4200 rads to reach this low level of function. They also reported that in seven parotid glands the flow was practically extinguished by this 550- to 700-rad dose, while three others required a much higher dosage. This same group of investigators (15) reported resting parotid flow rate responses for four additional patients receiving 200 rads per day. For three of the four patients, the flow rate fell to less than 60 mg/10 min after 600 rads. The fourth patient required 2600 rads to reach this low level.

It is thus clear that the human parotid gland is exquisitely sensitive to radiation and that the response is rapid and virtually complete. A single dose of 225 rads reduced the rate of resting parotid flow by one-half and a second dose of the same magnitude induced the virtual elimination of the remaining flow. It is evident that these changes are not rapidly reversible since these collections were made 24 hr after the radiation was administered. This deprivation of flow from serous elements explains the ropy, sticky, viscous nature of the residual oral fluid that is generally seen in these patients.

A further complicating consideration is that, if animal caries studies can be extrapolated to the human, this serous deprivation may specifically accelerate caries development. Cheyne (17) removed salivary glands selectively from rats and placed the animals on cariogenic or control diets. The operated animals with totally removed serous components showed a very high incidence of caries. Removing other combinations of glands produced an increased level of caries but it was much less marked. It was concluded that the animals developed caries in direct proportion to the amount of serous gland elements that was removed.

Summary. Resting parotid flow rate response to irradiation was evaluated in seven patients. The mean for pretreatment flow was 0.045 ml/min (SD = 0.023). Twenty-four hours after the first treatment (225 rads) this mean had decreased to 0.023 ml/min (SD = 0.022), and after the second treatment the mean was only 0.001 ml/min (SD = 0.002). Six of the seven patients exhibited no flow at all after 450 rads administered over 2 days, and the seventh flowed at a rate of only 0.005 ml/min. There was no detectable resting parotid flow from these patients after this time. These results confirm that the human parotid gland is highly sensitive to irradiation and suggest that virtually all of the oral fluid generated by the irradiated patient, even very early in the course of treatment, originates in glands other than the parotid.

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Effect of Calcium on Motility and Fertilization by Rat Spermatozoa *in Vitro* (39989)

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It is well established that Ca^{2+} ions effect both motility and fertilizing capacity in mammalian spermatozoa (1-3). However, it is apparently not known if these two aspects of sperm cell function have similar Ca^{2+} concentration requirements. Such a correspondence might be anticipated if maximum sperm motility was necessary for fertilization. Regarding this possibility, Yanagimachi (4) has described the motility of *in vitro* capacitated epididymal hamster spermatozoa as "extraordinarily active." On the other hand, Overstreet and Cooper (5) report that there is a marked reduction in motility among rabbit sperm in the isthmus of the oviduct.

Data are presented in this report concerning the effect of Ca^{2+} ions on motility and fertilizing ability of cauda epididymal rat spermatozoa in a chemically defined medium. While sperm motility is ordinarily appraised by subjective means, in the present study it was preferable to use a quantitative index of motility. For this purpose, a previously described (6) measure of sperm motility has been employed that relies on the turbulence generated in a suspension of highly motile spermatozoa.

Materials and methods. Sprague-Dawley rats from Charles River Breeding Laboratory were used in these experiments. Sperm cells and eggs were obtained and incubated in a manner similar to that described previously (6, 7). The incubation medium, modified Krebs-Ringer bicarbonate, was prepared to contain between 0 and 6.8 m*M* Ca^{2+}. The incubations were conducted in a humidified atmosphere of 5% CO_2 and 95% air at 37°.

An index of high sperm motility during incubation was provided by the number of turbulence swirls forming at the edge of a 0.1-ml suspension, which was covered with paraffin oil and contained 3×10^6 spermatozoa (6). The sperm cells had been obtained by sectioning a tubule in the cauda epididymis of a mature rat (body weight, 500 g). Sperm fertilizing ability was estimated from the number of fertilized eggs obtained after incubation for 13 to 14 hr with cauda epididymal rat spermatozoa. The eggs were removed from the medium with a fine glass pipet, stained with 0.25% lacmoid, and examined under a phase-contrast microscope (×250). Eggs were judged to be fertilized when they showed pronucleus formation and a fertilizing sperm tail.

Results. Figure 1 indicates that cauda epididymal spermatozoa failed to generate turbulence swirls when incubated in Krebs-Ringer bicarbonate medium lacking Ca^{2+}. This reveals that the cation is required for vigorous motility by rat sperm cells. An adverse effect on motility was also observed at Ca^{2+} concentrations higher than 1.7 m*M*. In medium with 3.4 m*M* Ca^{2+} there was vigorous motility for about 2 hr, however, turbulence declined rapidly and was not apparent after 4.5 hr. Both the maximal intensity and duration of turbulence in sperm suspensions with 5.1 m*M* Ca^{2+} were markedly reduced. Medium having 1.7 m*M* Ca^{2+} maintained sufficiently high sperm motility to permit some turbulence in the suspension for at least 15 hr. It should be noted that sperm motility could be observed in these suspensions for several hours after they had ceased to show turbulence.

Sperm fertilizing capacity was sensitive to Ca^{2+} levels in the medium (Fig. 2). At Ca^{2+} concentrations of 2.6 and 3.4 m*M* the respective fertilization rates were 83% (44/53) and 91% (59/65). In medium containing 1.7 and 6.8 m*M* Ca^{2+} the rates of fertilization were 31% (17/55) and 13% (3/23), respectively. At 6.8 m*M* Ca^{2+} the medium had a slightly cloudy appearance, indicating that saturation had been reached.

Discussion. These results show that motility and fertilization by epididymal rat spermatozoa differ in their dependence on Ca^{2+} ions. Evidently the maximization of sperm

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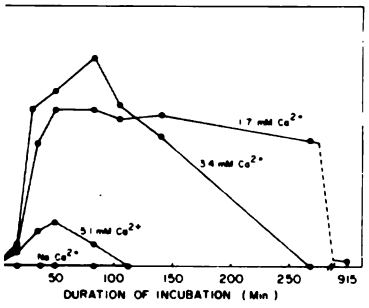
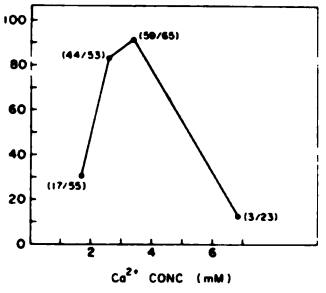


High motility in suspensions of cauda epi- t spermatozoa at different Ca^{2+} concentra- rm motility is related to the number of swirls that form at the edge of a 0.1-ml , containing 3 × 10^6 spermatozoa, during



Effect of Ca^{2+} ions on fertilization *in vitro* . The eggs were incubated with epididymal ıa (0.5 × 10^6 sperm/ml) in a modified ger bicarbonate medium containing 10 mg /ml. The number of fertilized eggs and the er are given in parentheses.

s not necessary for fertilization in ies. It should be noted, however, conclusion is based on estimates of n the whole sperm population, and eivable that sperm cells responsible zation actually had higher motility data indicate.

s have been reported by Barros associates (8) which suggest that ıs in the pattern and vigor of sperm are not essential for fertilization. ave shown that noncapacitated ig spermatozoa can penetrate zona -free hamster eggs *in vitro* when ome reaction is induced by placing :rm between a coverslip and glass a period of minutes. Guinea pig zoa usually require 12 to 18 hr of ın under the conditions used in this study before they undergo the acrosome reaction and express fertilizing ability (8, 9). Fusion between the plasmalemma and outer acrosomal membrane, which is the basis of the mammalian acrosome reaction (10), is evidently the only impediment to fertilization by noncapacitated sperm cells. Other changes that might be associated with prolonged incubation, such as changes in respiration and motility, apparently have only secondary significance.

There is good agreement between the fertilization rate data in Fig. 2 and the results presented by Miyamoto and Ishibashi (3). However, the comparison is limited since media with Ca^{2+} concentrations between 1.71 and 5.13 m*M* were not examined in the latter investigation. From the incidence of the acrosome reaction among guinea pig sperm after 4 hr of incubation (11), medium with 3 m*M* Ca^{2+} is twice as effective as that with only 1 m*M* Ca^{2+} for promoting this process. The occurrence of a similar dependence on Ca^{2+} ions in rat spermatozoa would apparently account for the present results, which show that rat sperm cells display high fertilizing capacity at 2.6 and 3.4 m*M* Ca^{2+}. On the other hand, the reported differences in fertilization rate could also reflect Ca^{2+} effects on rat ova. The present observations serve to indicate, however, that the optimization of sperm motility may not be mandatory for fertilization.

Summary. Motility and the expression of fertilizing ability by cauda epididymal rat spermatozoa, incubated in a chemically defined medium, were demonstrated to differ in their dependence on Ca^{2+} ions. Motility was optimal at 1.7 m*M* Ca^{2+}, whereas fertilization rates were significantly higher at 2.6 and 3.4 m*M* Ca^{2+}. These results are interpreted as indicating that sperm motility may not have to be maximized for fertilization to occur.

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ption of Mercury from Ligated Segments of the Rat Gastrointestinal Tract[1] (39990)

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derstanding of the mechanisms in- n the absorption of different forms ury by the gastrointestinal tract is ete, although significant progress n made for inorganic mercury (1). n and Cross (2) and Cikrt (3, 4) the uptake of inorganic mercury by l and ileal intestinal everted gut hagian *et al.* (5, 6) examined the in isolated strips of intestinal seg- nd in perfused everted intestinal d Cikrt (7) studied the absorption anic mercury from ligated intestinal s in the rat. General conclusions the uptake of inorganic mercury by ntestine exhibits the characteristics sive transport mechanism and that, n only a small percentage is ab- the duodenal and ileal sections are nal sites of absorption. Methylmer- wever, is readily absorbed (8–10), orption sites have not been deter-

ts of our attempts to kinetically an- ercury transport in the rat suggested hylmercury was absorbed from two e large gastrointestinal compart- he stomach appeared to be a major bsorption (unpublished data). Con- y, this study was conducted to de- the major sites of methylmercury on in the rat gastrointestinal tract compare the sites of organic and c mercury absorption.

ials and methods. Fifty mature male -Dawley rats averaging 430 g were y assigned to 10 experimental nd anesthetized with metofane after night fast. The peritoneal cavity was y exposed and the intestine was ligated to form three 8- to 10-cm l segments, a stomach compartment, and a small intestinal segment for bile collection. Ligatures were placed at the pyloric-duodenal junction; approximately 4, 14, 30 to 40, and 40 to 50 cm distal to the pyloris; and approximately 2 and 12 cm proximal to the ileocecal junction. Before the second ligature on the segment to be dosed was closed, a needle was inserted into the lumen which passed through the ligature and into the segment. The isotope was injected after the ligature was secured. The incision was closed with wound clips and the animals were allowed to recover from the anesthetic under heat lamps.

Half of the rats were each dosed with 5 μCi of methylmercury chloride (CH_3-$^{203}HgCl$, 2.5 mCi/mg) either in the stomach, duodenal segment, jejunal segment, ileal segment, or intravenously; and the other half were similarly injected with 20 μCi of $^{203}HgCl_2$ (3.9 mCi/mg). The mercury compounds, dissolved in 0.5 *M* HCl, were diluted about 1:500 with physiological saline and 0.5 ml was injected into each segment. Doses were administered intravenously by tail vein and by gavage to the stomach after completion of surgery. One milliliter of blood was collected at 1, 2, and 4 hr after dosing. After 4 hr the rats were anesthetized and decapitated, and tissues were collected for analysis. The length of gastrointestinal segments was also measured.

Blood, liver, kidneys, stomach, and intestinal segments were counted in a deep-well automatic gamma spectrophotometer; the remaining carcass of each rat was counted in a large-volume tissue counter containing two 3 × 3-in. NaI crystals connected to a Packard pulse height analyzer and scaler. Total absorption was determined from the activity in blood, liver, kidneys, and carcass, and total endogenous mercury reappearing in the gastrointestinal tract was determined from the activity in the nondosed segments.

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Analysis of variance was applied to the data, and differences between means were determined by Student-Newman-Keul's multiple-range test.

Results. The concentration (% dose/ml) of mercury in the blood 1, 2, and 4 hr after dosing with methylmercury ($CH_3{}^{203}HgCl$) or inorganic mercury ($^{203}HgCl_2$) is shown in Table I. As expected, blood concentrations of methylmercury were greater ($P < 0.01$) than those of inorganic mercury for all dosing methods. One- and two-hour methylmercury blood concentrations of rats dosed in different gastrointestinal segments were not different, but the blood concentration of methylmercury at 4 hr was slightly higher ($P < 0.05$) from rats dosed in the duodenal segment (the segment of greatest absorption). There was no correlation between segment absorption and blood concentrations of inorganic mercury.

Total absorption, GI retention, and excretion of endogenous mercury into the gastrointestinal tract are shown in Table II. The duodenal segment absorbed over 80% of a local methylmercury dose, greater ($P < 0.01$) than any other segment. Nearly 60% of a local methylmercury dose was absorbed from the stomach, not statistically different from the ileal segment. Absorption from the jejunum was 35%, less ($P < 0.01$) than any other segment. Gastrointestinal retention was inversely proportional to absorption. Significant differences in absorption of inorganic mercury between gastrointestinal segments were not detectable.

Total endogenous excretion of methylmercury into the gastrointestinal tract was proportional to absorption, but excretion as a percentage of dose was significantly less ($P < 0.01$) when the dose was administered in the jejunal segment. However, when the data were expressed as a percentage of the absorbed dose rather than as a percentage of the total dose, no differences were seen in excretion between sites of absorption.

Table III lists the endogenous excretion of mercury into the intestinal segments after intravenous injections of methylmercury chloride or mercury chloride. More ($P < 0.01$) of both forms of mercury was excreted into the first duodenal segment (which contained all bile excreted during the experiment) per unit length than any other segment. This was presumably due primarily to bile excretion. Significant amounts of mercury were also excreted into other intestinal segments; the duodenal segment was the optimal site for intestinal excretion. In contrast to mercury absorption from the intestine, mercury excretion by intestinal tissue was the same for both forms of mercury per unit length, and the excretion of mercury in bile from inorganic mercury was greater ($P < 0.05$) than that of methylmercury.

Discussion. Methylmercury was readily absorbed from all segments of the gastrointestinal tract in the following pattern: duodenum > stomach = ileum > jejunum.

TABLE I. THE CONCENTRATION OF ^{203}Hg ($CH_3{}^{203}HgCl$ AND $^{203}HgCl_2$) IN THE BLOOD 1, 2, AND 4 hr FOLLOWING INTRAVENOUS INJECTIONS AND INJECTIONS INTO LIGATED SEGMENTS OF THE GASTROINTESTINAL TRACT (% DOSE/g ± SE).

Segment	1 hr	2 hr	4 hr
	Methylmercury		
iv	5.10 ± 0.36*, a	4.57 ± 0.48[a]	4.05 ± 0.70[a]
Stomach	0.61 ± 0.08[b]	1.08 ± 0.13[b]	1.25 ± 0.11[b]
Duodenum	0.41 ± 0.04[b]	0.98 ± 0.06[b]	2.10 ± 0.04[c]
Jejunum	0.47 ± 0.06[b]	0.82 ± 0.12[b]	0.91 ± 0.11[b]
Ileum	0.42 ± 0.08[b]	0.86 ± 0.18[b]	1.22 ± 0.34[b]
	Mercury chloride		
iv	1.90 ± 0.11[a]	1.40 ± 0.05[a]	1.16 ± 0.06[a]
Stomach	0.014 ± 0.006[b]	0.012 ± 0.007[b]	0.019 ± 0.009[b]
Duodenum	0.011 ± 0.005[b]	0.018 ± 0.006[b]	0.019 ± 0.006[b]
Jejunum	0.006 ± 0.003[b]	0.012 ± 0.005[b]	0.025 ± 0.014[b]
Ileum	0.014 ± 0.007[b]	0.014 ± 0.006[b]	0.013 ± 0.005[b]

* Mean ± SE. Means with the same subscript in the same vertical row are not different at the 5% level of significance.

LE II. GASTROINTESTINAL RETENTION, ABSORPTION, AND ENDOGENOUS EXCRETION OF MERCURY AFTER INJECTIONS OF METHYLMERCURY OR MERCURY CHLORIDE INTO LIGATED SEGMENTS.

dose	GI retention (% dose)	Absorption[a] (% dose)	Total endogenous excretion[b]	
			% Dose	% Absorbed
		Methylmercury		
ch	35.9 ± 2.4[*,c]	58.6 ± 2.0[c]	3.60 ± 0.28[c]	6.14 ± 0.96[c]
:num	15.8 ± 1.1[d]	83.1 ± 2.3[d]	4.65 ± 0.22[c]	5.60 ± 0.42[c]
ım	58.4 ± 3.8[e]	34.8 ± 3.0[e]	2.18 ± 0.25[d]	6.26 ± 1.44[c]
	31.9 ± 3.2[c]	54.6 ± 6.4[c]	3.34 ± 0.30[c]	6.12 ± 1.10[c]
		Mercury chloride		
ch	91.1 ± 2.2[c]	3.66 ± 1.18[c]	0.314 ± 0.138[c]	8.85 ± 2.46[c]
:num	90.0 ± 1.1[c]	2.33 ± 0.66[c]	0.218 ± 0.046[c]	10.64 ± 3.05[c]
ım	90.7 ± 1.1[c]	2.00 ± 0.89[c]	0.134 ± 0.041[c]	9.92 ± 2.63[c]
	95.6 ± 2.4[c]	1.91 ± 0.93[c]	0.197 ± 0.060[c]	10.98 ± 3.68[c]

f activity in blood, liver, kidneys, and carcass.
activity in all nondosed gastrointestinal segments.
± SE. Means with the same subscript in the same vertical row are not different at the 5% level of
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ABLE III. ENDOGENOUS EXCRETION OF MERCURY INTO THE GASTROINTESTINAL TISSUE AFTER INTRAVENOUS INJECTIONS OF EITHER METHYLMERCURY OR MERCURY CHLORIDE.

ient	Methylmercury		Mercury chloride	
	% Dose/cm	% Dose/g	% Dose/cm	% Dose/g
(bile)	0.081 ± 0.008[*,a]	0.248 ± 0.027[a]	0.169 ± 0.024[a]	0.613 ± 0.990[a]
	0.036 ± 0.003[b]	0.444 ± 0.017[b]	0.078 ± 0.015[b]	1.410 ± 0.230[b]
	0.023 ± 0.002[b]	0.340 ± 0.025[a]	0.029 ± 0.002[c]	0.520 ± 0.041[a]
	0.025 ± 0.005[b]	0.283 ± 0.015[a]	0.042 ± 0.006[c]	0.661 ± 0.082[a]
		0.121 ± 0.020[c]		0.191 ± 0.017[c]
ɔn		0.171 ± 0.015[c]		0.321 ± 0.038[b]

± SE. Means with the same subscript in the same vertical row are not different at the 5% level of
:.

: mercury was not readily ab-
nd no significant difference existed
segments.

;nificance of the high absorption of
:rcury from the stomach is difficult
ıte, since the surface area of the
may differ from that of intestinal
and the emptying time of methyl-
from the stomach may be very
he normal animal. Methylmercury
ıal diet is probably protein bound
er than ionic and may not be as
ɔsorbed from the stomach until the
ıave been digested. Thus the resi-
ıe of the readily absorbable form
lmercury could reduce absorption
omach. It is apparent, however,
ıbsorption of methylmercury from
ıch is likely to contribute a sizable
f total methylmercury absorption.
ttern of metal absorption from the
stinal tract differs for various ele-
ments and even for different chemical forms of the same element. This study demonstrates that, unlike methylmercury, inorganic mercury was not readily absorbed from the stomach or other intestinal segments. Thus methylmercury was absorbed more readily from all segments of the gastrointestinal tract studied, a relationship which has been observed many times in whole animal studies (8–10). Whanger *et al.* (11) found that inorganic and organic selenium were absorbed equally from the gastrointestinal tract and that essentially no selenium was absorbed from the stomach. Among other metals studied only copper is readily absorbed from the stomach (12).

The major route of mercury excretion is via the feces, and both methylmercury (13, 14) and inorganic mercury (15) are excreted in the bile. Autoradiographic studies by Berlin and Ullberg (16) have also shown an accumulation of intravenously injected mer-

curic chloride in the mucosa of the entire intestinal tract. Our data indicate that both methylmercury and inorganic mercury are excreted by intestinal tissue as well as in bile.

Summary. The ligated segment technique was used to compare the gastrointestinal absorption of methylmercury and mercury chloride in the rat. Methylmercury was more readily absorbed (15–35 times greater, depending on the absorption site) than inorganic mercury from all ligated segments. The relative order of methylmercury absorption from ligated segments was as follows: duodenum > stomach = ileum > jejunum. Differences in absorption of inorganic mercury between gastrointestinal segments were not observed. Endogenous excretion of both forms of mercury into intestinal tissue were equal. Our data indicate that the absorption of methylmercury from the stomach is significant relative to other parts of the gastrointestinal tract and that the stomach must be considered a major site of absorption.

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Radiolabeling of Lymphocytes and Tumor Cells with ^{111}Indium[1] (39991)

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ction. The recirculation of lym- from blood to lymph has been in all mammalian species investi- 2). While the major pathways of te circulatory patterns have been ı mice, rats, and sheep, only pre- lata are available regarding lym- ecirculation in man (3-5). One r this difficulty has been the lack ɔisotopic label useful in humans. ymphocyte recirculation studies d extensively on radiolabeling of tes with [^{51}Cr]sodium chromate 6, 7). Unfortunately, this label nt deficiencies which make it less nan. ^{51}Cr is a low-energy γ photon nly 9% of its energy being emitted makes ^{51}Cr less suitable for exter- ing by a γ camera or rectilinear Because of its relatively weak γ high doses of ^{51}Cr would have to ı labeling lymphocytes for use in canning. The use of high-dose ^{51}Cr tion with its half-life of 27.8 days ɔose subjects to unnecessarily high radiation (300 μCi of ^{51}Cr results ı dose of 10 rads and a total body .12 rads). In our hands, doses of e range of 100 μCi/10^8 lympho- lt in a loss of 40-50% of the cells. n, ^{51}Cr does not label lymphocytes and any migration studies may reflect the circulatory properties cyte populations (6, 8). For these e investigated other radioisotopes or lymphoid cells. Our choice of is based on the work of McAfee ır (9, 10), Thakur *et al.* (11), and '. (12), who labeled polymorpho- ukocytes with ^{111}indium for use in abscesses.

Animals. Balb/c mice, 6-10 age, were purchased from a commercial supplier (Cumberland View Farms, Clinton, Tenn.). Male Lewis rats (150-250 g) were obtained from Microbiological Associates (Walkersville, Md.) and splenectomized at the time of thoracic duct cannulation.

Lymphocytes. Balb/c thymus was used as a source of T cells. B cells were obtained from the lymph node and spleen of Balb/c nude mice from our own colony. Rat thoracic duct lymphocytes were obtained by cannulation of the thoracic duct using the method of Bollman *et al.* (13). Macrophages were obtained from the peritoneum or spleen by the adherence of these cell suspensions to glass. One hundred million spleen or peritoneal exudate cells were placed in glass petri dishes in minimal essential medium (MEM) with 10% fetal calf serum (FCS) and allowed to incubate at 37° for 30 min. The supernatant was discarded and adherent cells were removed with a silicone rubber policeman. This procedure was repeated and macrophage purity was found to be >95% by morphologic criteria.

Radioisotopes. ^{111}Indium chloride (^{111}InCl) was obtained from Medi-Physics, Inc. (Emeryville, Calif.). It has a half-life of 67 hr and emits γ photons at 173 keV (89%) and 247 keV (93%). ^{51}Cr (as sodium chromate) was obtained from New England Nuclear (Boston, Mass.). It has a half-life of 27.8 days and emits a single γ photon at 320 keV (9%).

The preparation of ^{111}indium-oxine complex. The method of Thakur *et al.* was used (11). Briefly, 3 mCi of ^{111}InCl at pH 1-3 was added to an equal volume of sterile distilled water followed by 200 μl of 0.3 *M* acetate buffer, pH 5. One hundred and fifty micrograms of 8-hydroxyquinoline (oxine; Sigma Chemical Co., St. Louis, Mo.) was dissolved in absolute ethanol (Commercial Solvents Corporation, Terra Haute, Ind.) and added to the mixture. The tube

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was mixed well and allowed to stand at room temperature for 15 min. An equal volume of chloroform (Baker Chemical Company, Phillipsburg, N.J.) was then added. The solution was mixed and allowed to stand at room temperature for 10 min. The water layer was discarded and the chloroform evaporated in a boiling water bath. The remaining ^{111}indium-oxine chelate was dissolved in 100 μl of absolute ethanol and diluted to 500 μl with normal saline prior to use. In all preparations of the ^{111}indium-oxine complex, excess oxine was added to saturate any cadmium impurities. Under these conditions, approximately 75–85% of the original γ activity of the ^{111}InCl is extracted as the chelated ^{111}indium-oxine complex (^{111}InOx). This was determined by comparing counts per minute (cpm) of an aliquot of the original ^{111}InCl (from a known volume). By this means, we can determine the number of microcuries of ^{111}InOx added to any individual cell suspension.

Results. The effect of ^{111}indium-oxine dose. Figure 1 demonstrates that with the addition of increased amounts of ^{111}InOx to a constant number of cells in a standard volume, the number of counts per cell increases from 2.5×10^5 cpm/10^7 cells at 2.5 μCi to 2.5×10^6 cpm/10^7 cells at 10μCi. While the counts per minute per 10^7 cells increases steadily, it is not exponential and the difference in labeling efficiency at 50 and 100 μCi is increased by only 24% for the 100% increase in available label. The viability of the cells (as measured by trypan blue) remains relatively constant at >90%, indicating that the ^{111}InOx complex is relatively nontoxic. Labeling with ^{111}InOx that had passed through four half-lives yielded identical viabilities, reflecting the nontoxic nature of the decay product. Cell viability has also been confirmed by *in vivo* lymphocyte migration studies (see below).

Effect of cell concentration and ^{111}InOx dose on labeling efficiency. Efficiency of labeling with ^{111}InOx is related to two variables, the cell concentration and ^{111}InOx dose. Figure 1 describes the effect of changing the ^{111}InOx dose added to a standard cell concentration. If the ^{111}InOx dose is kept constant and the cell concentration is increased or decreased above or below 10^7 cells/ml, labeling efficiency per cell decreases. Though optimal labeling is achieved at 10^7/ml, our labeling procedure is standardized at a cell concentration of 5×10^7/ml for practical reasons. The difference in labeling per cell at this higher cell concentration is insignificant.

Effect of incubation time and temperature. Figure 2 demonstrates that the advantage gained in labeling efficiency per cell by longer incubation times is lost in viable cell recovery. Because of these observations, we have opted for a 15-min incubation time. Labeling at 22 or 37° produced almost identical labeling efficiencies and viable cell recoveries.

Effect of medium and fetal calf serum. Cells can be labeled in PBS, MEM, and tissue culture media 199 or RPMI-1640, providing 10% FCS is added as a media supplement. Media not containing FCS result in 30% less efficient labeling. RPMI-1640 with 10% FCS gave the most consistent and highest labeling efficiency. In 12 successive experiments, the addition of 10 μCi of ^{111}InOx to 5×10^7 lymph node lymphocytes in 0.5 ml of RPMI-1640 with 10% FCS and 20 m*M* Hepes resulted in $2.71 \times 10^6 \pm 0.5 \times 10^6$ cpm/10^7 cells. Similar results were obtained when labeling tumor cells or spleen cells in RPMI-1640.

Cell type Labeled. We have observed efficient labeling of adherent and nonadherent (>95% macrophages) spleen cells, thymic and lymph node cells from normal mice, and lymph node cells from nude Balb/c mice, as well as lymphocytes from the thoracic duct of rats. DBA/2 mastocytoma cells and human cell lines [EB-33, H494, 14975 (prostate); malme and miwa (melanoma)] are also efficiently labeled and release ^{111}InOx in cytotoxicity assays (14) (Wiltrout and Frost, unpublished).

Spontaneous release. Table I summarizes the spontaneous release of ^{111}InOx and ^{51}Cr from lymphocytes and DBA/2 mastocytoma cells incubated in culture for 24 hr. While the spontaneous release of ^{111}InOx from lymphocytes is more gradual than that observed with ^{51}Cr, the total release is comparable at 24 hr. However, this is not true with mastocytoma cells where the spontaneous release of ^{111}InOx is at least fourfold

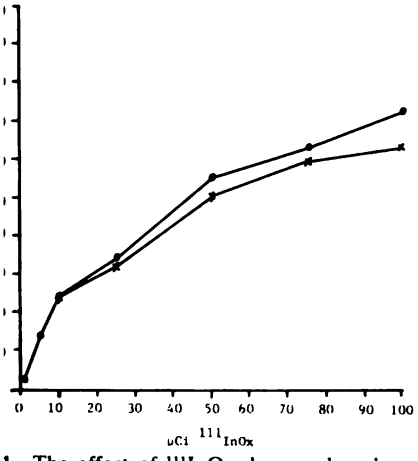
an that of ^{51}Cr at 24 hr. The reason mastocytoma cells should release x at a much slower rate than lympho- s currently unclear. Similar low spon- is release has been observed with hu- mor lines (14).

ilization. The lysis of ^{111}InOx-labeled 2 mastocytoma cells by cell rupture esults in the release of isotope in an wn form. The addition of released e to unlabeled DBA/2 mastocytoma r lymphocytes results in less than 1% taken up by these cells. This implies ace the ^{111}InOx complex is taken up by cells, it is altered sufficiently so as to render it nonreutilizable.

TABLE I. PERCENTAGE SPONTANEOUS RELEASE OF ^{51}Cr AND ^{111}In FOR LYMPHOCYTES AND TUMOR CELLS.[a]

Time (hr)	Lymph node lymphocytes		DBA/2 mastocytoma cells	
	^{51}Cr	^{111}InOx	^{51}Cr	^{111}InOx
2	13.5	7.5	5.3	1.3
4	21.6	13.6	9.5	2.2
6	32.7	17.2	12.6	2.6
24	67.2	59.8	24.8	6.3

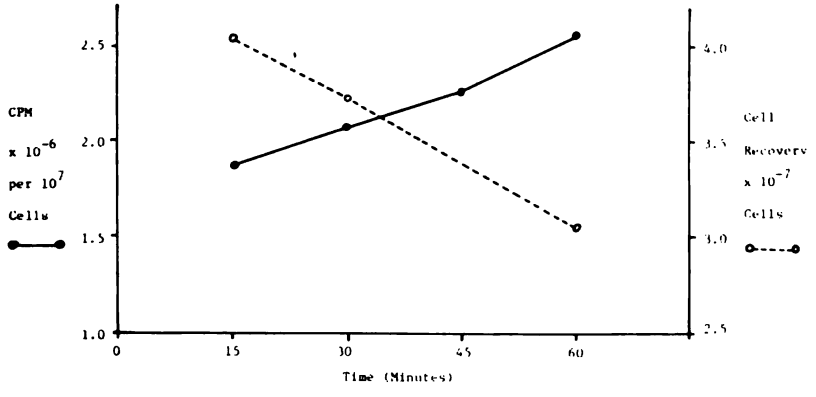
[a] Five × 10^7 lymphocytes or DBA/2 mastocytoma cells were labeled with either ^{51}Cr or ^{111}InOx and placed in culture. Culture samples were assessed for spontaneous release of label at varying times thereafter.



1. The effect of ^{111}InOx dose on lymphocyte Increasing amounts of ^{111}InOx were added to lymphocytes in 0.5 ml of RPMI with 10% l 20 m*M* Hepes buffer. The cells were incu- r 15 min at 37°. O——O, Expt. 1; ×——×,

Membrane vs cytoplasmic labeling. To demonstrate whether ^{111}InOx labeled membrane or cytoplasm, labeled cells were dispersed in a cell rupturing pump (15). The cellular constituents were separated by differential centrifugation (15, 16) and less than 10% of the label was found to be associated with membrane. In addition, attempts at labeling purified membrane fragments resulted in poor labeling efficiency.

Comparison of ^{111}indium-oxine and ^{51}Cr as lymphocyte labels. Table II summarizes the labeling efficiency of ^{51}Cr and ^{111}InOx on lymph node, spleen, and thymic cells, utilizing threefold more ^{51}Cr than ^{111}InOx (30 μCi of ^{51}Cr as sodium chromate and 10 μCi of ^{111}InOx). The increase in labeling efficiency with ^{111}InOx is 47-fold, 65-fold,



. The effect of incubation time on labeling efficiency. Ten microcuries of ^{111}InOx was added to 5 × 10^7 tes in 0.5 ml of RPMI with 10% FCS. The mixture was allowed to incubate for 15, 30, 45, or 60 min.

and 13.3-fold with lymph node cells, spleen cells, and thymic cells, respectively.

The recirculation of ^{111}indium–oxine lymphocytes in the rat. ^{111}InOx-labeled rat thoracic duct lymphocytes (TDL) recirculate from blood to lymph and distribute to organs in a manner almost identical to that seen with TDL labeled with other isotopes (Table III). In these experiments, cells were labeled with 20 μCi of ^{111}InOx, a dose sufficient for detection by nuclear scanning (17). Doses of ^{111}InOx in excess of 100 μCi/10^8 result in a normal initial organ distribution (useful in scanning) but diminished recirculation of the cells.

Discussion. Optimal conditions for labeling lymphocytes, macrophages, and tumor cells with ^{111}InOx have been outlined. The ^{111}InOx primarily labels cell cytoplasmic components, probably by passive diffusion of the highly lipophilic metal oxine complex (11). The spontaneous release of ^{111}InOx for cells is low and the label released is nonreutilizable.

^{111}InOx offers several advantages over other labels in studies of lymphocyte recirculation and tumor cell cytotoxicity. It has a short half-life (67 hr), far shorter than that of ^{51}Cr (27.8 days) and longer that that of ^{99}technetium (6 hr). Because it emits two γ photons, it can be detected by a γ camera and rectilinear scanner (17). A variety of cells are labeled to high specific activities with minimal loss in viability, and finally, labeled cells show significantly lower spontaneous release of label when compared with ^{51}Cr.

These observations, taken together with evidence that ^{111}InOx-labeled lymphocytes recirculate in rats and distribute to lymphoid organs normally, make it of potential value in the study of lymphocyte recirculation in man. The low spontaneous release from human and animal tumor cells may provide the long-term isotope release assay needed in the study of cytotoxicity to human tumors.

TABLE II. COMPARISON OF ^{51}Cr AND ^{111}InOx AS LYMPHOCYTE LABELS.[a]

	Counts per minute per 10^7 cells		Ratio of ^{111}InOx to ^{51}Cr
	^{51}Cr	^{111}InOx	
Lymph node cells	5.8×10^4	275×10^4	47
Spleen cells	6.9×10^4	451×10^4	65
Thymocytes	13×10^4	173×10^4	13.3

[a] Five $\times$ 10^7 lymph node, spleen, or thymic lymphocytes were labeled with 30 μCi of ^{51}Cr or 10 μCi of ^{111}InOx and the cpm/10^7 cells were assessed after incubation at 37° for 15 min in RPMI and 10% FCS.

Summary. ^{111}Indium when complexed

TABLE III. RECIRCULATION AND ORGAN DISTRIBUTION OF ^{111}InOx-LABELED RAT THORACIC DUCT LYMPHOCYTES.[a]

(a) Recirculation				(b) Organ distribution (30 hr)	
Time (hr)	Volume of lymph (ml)	CFL[b]	TDL[c]		
0-6	16 ± 5	0.58 ± 0.14	1.99 ± 1.3	Lung	0.58 ± 0.08
6-12	25 ± 7	1.14 ± 0.46	6.03 ± 2.6	Liver	17.56 ± 3.66
12-18	16 ± 3	0.35 ± 0.40	6.00 ± 1.63	Thymus	0.84 ± 0.38
18-24	24 ± 5	0.31 ± 0.13	5.11 ± 0.56	Small bowel	7.84 ± 2.50
24-30	23 ± 4	0.43 ± 0.10	3.38 ± 0.37	Femur	0.90 ± 0.28
				Kidney	0.64 ± 0.09
				Testes	0.04 ± 0.01
				Peripheral lymph nodes	4.89 ± 0.82
				Mesenteric lymph nodes	6.08 ± 1.82
				Whole blood, 1 ml	0.77 ± 0.68
				Total recovery (in thoracic duct lymph and sampled organs)	65.39 ± 6.56

[a] Mean ± SD (expressed as percentage of counts injected) of recovered ^{111}InOx activity from four adult splenectomized rats. (a) Time course of appearance of recirculating cells in the thoracic duct. (b) Organ distribution of labeled cells at 30 hr after intravenous injection. Cells were labeled with 20 μCi of ^{111}InOx/10^8 cells.

[b] Thoracic duct lymphocytes.

[c] Cell-free lymph.

with oxine is an efficient label of lymphocytes and tumor cells. ^{111}Indium–oxine complex (^{111}InOx) is not reutilizable, making it useful for short-term lymphocyte recirculation studies. The spontaneous release of ^{111}InOx from tumor cells is very low, making it a useful isotope in cytotoxicity studies. ^{111}InOx emits two γ photons detectable by nuclear scanning, making this label of potential use in the study of lymphocyte migration in man.

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Independence of Bile Acid and Ouabain Hepatic Uptake: Studies in the Newborn Rat[1] (39992)

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It is generally accepted that there are at least three separate active transport systems in the liver for the overall transfer of organic compounds from plasma into the bile (1). One transport system is responsible for the transfer of organic anions such as sulfobromophthalein and bilirubin, the second for organic cations such as procainamide ethobromide and *d*-tubocurarine, and the third for neutral organic compounds or steroids such as ouabain.

It is not known if bile acids are transported by one of the aforementioned processes or if yet another transport mechanism for bile acids exists. Sperber (2) originally suggested that a common mechanism existed for the hepatic excretion of all organic anions. However, it was later shown that Corridale sheep, which cannot excrete sulfobromophthalein and other organic acids into bile, can excrete bile acids normally into bile (3). This indicates that bile acids are excreted by a process different than that of most organic acids. Bile acids have been shown to inhibit the uptake and biliary excretion of ouabain (4), and thus it has been suggested that bile acids might be excreted by the steroid transport system.

We have recently shown that, while the liver of the adult rat has a marked ability to concentrate ouabain, the liver of the newborn rat has an absolute inability to concentrate ouabain (5). However, while the liver of the newborn rat is not as efficient in excreting organic anions as the adult, it does have the ability to concentrate them (6). Therefore, it was of interest to determine if bile acids are excreted as efficiently in the newborn as the adult rat. If the liver of the newborn rat was found to have the ability to concentrate the bile acids, this would suggest that bile acids are not transported by the same process that transports ouabain.

Material and methods. Rats of various ages were used throughout the study. The rats were born in our laboratory and were produced by mating untreated Sprague-Dawley (Bio-Lab, White Bear, Minn.) rats. The mother and offspring were kept in "shoebox cages" for 1 month before separation. The rats had free access to food and water at all times.

[^{3}H]Taurocholic acid (New England Nuclear, Boston, Mass.) and unlabeled taurocholic acid (Calbiochem, San Diego, Calif.) were mixed to give the appropriate specific activity. In the first study the [^{3}H]taurocholic acid (30 mg/kg) was injected (10 mg/kg) into the distal portion of the femoral vein. Fifteen minutes later, a blood sample was obtained by cardiac puncture under ether anesthesia. Heparin was used as the anticoagulant. In the second study the [^{3}H]taurocholic acid was injected similarly, but the blood sample was obtained at 2, 5, 10, 20, or 30 min thereafter. The rats were sacrificed, and the liver and small intestine were removed. As a measure of the biliary excretion of taurocholic acid, the small intestine was homogenized with 3 ml of saline in a Brinkmann Polytron homogenizer (Luzern, Switzerland). This method was used to estimate biliary excretion because it was not feasible to cannulate the bile duct of small rats and measure biliary excretion directly. This technique for estimating biliary excretion was verified by comparing the amount of ^{3}H in the small intestine of bile-duct-ligated and control (adult) rats 2 min after iv administration of [^{3}H]taurocholic acid; the concentration in bile-duct-ligated rats was 4.6% of the control value. Aliquots of plasma (50–250 μl), intestinal homoge-

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te (500 μl), and liver (approximately 500 ;) were oxidized with a Packard Model 6 Tri-Carb sample oxidizer, and radioac- ity was estimated with a Packard Tri- rb liquid scintillation spectrometer (Pack- l Industries, Downers Grove, Ill.). ıenching was determined by using auto- ıtic external standardization.

The 7-day-old animals were compared to : 39-day-old animals by Student's *t* test described by Steel and Torrie (7).

Results. The plasma concentration of tau- :holic acid 15 min after iv administration) mg/kg) in rats of various ages is depicted Fig. 1. In rats 20 days of age and older : plasma concentration of taurocholic acid approximately 6 μg/ml. The plasma con- ıtration is more than fourfold higher in : 10-day-old rats. There is a marked de- :ase in the plasma concentration of tau- :holic acid 15 min after administration as : rats increase in age from 5 to 20 days. ıe plasma concentration of taurocholate the 5-day-old rats is significantly lower ın that in the 10-day-old rats.

In an attempt to determine why the ısma taurocholate levels were higher in : newborn rats than in the adult rats 15 n after administration, the concentration taurocholic acid in the plasma, the ıount of taurocholic acid contained in the er, and the amount excreted into the estine were measured at 2, 5, 10, 20, d 30 min after administration to 7- and -day-old rats. Figure 2 illustrates that the ısma level of taurocholic acid was already nificantly higher in the 7-day-old than in : 39-day-old rats at 2 min after adminis- ıtion, suggesting a larger volume of distri-

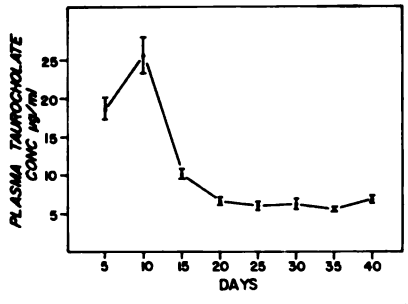

FIG. 1. Plasma taurocholic acid concentration 15 ı after iv administration (30 mg/kg). Each value resents the mean ± SE of eight rats.

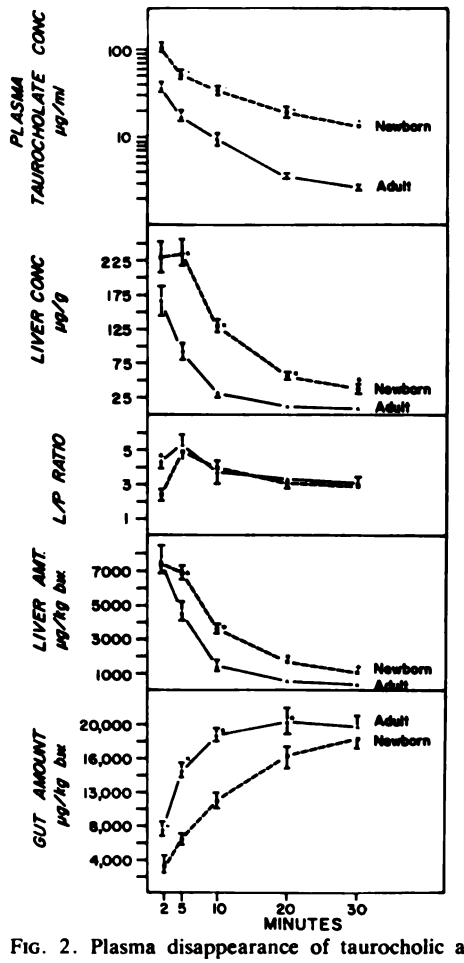

FIG. 2. Plasma disappearance of taurocholic acid (30 mg/kg), liver concentration, liver content, liver/plasma taurocholic acid concentration, and biliary excretion of taurocholic acid in 7- and 39-day-old rats. Each value represents the mean of 6–10 rats. Asterisk indicates the values are significantly different from controls, $P < 0.05$.

bution. The difference between the newborn and adult became greater at the longer time intervals. The concentration of taurocholate in the liver of the newborn rat was significantly higher than that in the adult at 5 min after administration and at all times thereafter (Fig. 2). However, since the liver of the newborn rat comprises only 2.9% of its body weight while the liver of the older rat is 4.6% of its body weight, the difference between the quantity of taurocholic acid in the liver of the newborn and the adult is less than the difference in concentration.

However, there is significantly more taurocholic acid in the liver of the newborn than the adult rat at 5 min after administration and all times thereafter (Fig. 2). Another index of overall hepatic excretory function is the ratio of concentration of taurocholic acid in the liver to its concentration in the plasma. The value in the newborn rat was significantly lower than in the older animals at the 2-min interval but was similar to the adult rat at all times thereafter. The amount of taurocholic acid detected in the small intestine was used as a measure of its biliary excretion. The newborn rats excreted significantly less of the taurocholate into the intestine than the adults at all times after administration except at 30 min (Fig. 2).

Discussion. Similar to the neutral organic compound ouabain (5, 8) and the organic anions sulfobromophthalein (BSP) and indocyanine green (ICG) (6), taurocholic acid is not handled as efficiently by the liver of the newborn rat as it is by that of the adult. The time course for development of the systems responsible for the transfer of taurocholic acid is similar to that for ouabain (5), BSP, and ICG (6). The overall excretory capacity of the liver appears to be mature by 20 days of age. Interestingly, the liver of the 5-day-old rat appears to be more efficient in handling an exogenous load of taurocholic acid than the liver of the 10-day-old rat; similar results were obtained with ouabain (5, 8) as well as with BSP and ICG (6).

The mechanism for the delayed disappearance of taurocholic acid from the plasma of the newborn was examined by quantitating the amount of taurocholate in the plasma and liver and that excreted into the bile at various times after iv administration. At 2 minutes the concentration of taurocholate in the plasma was already higher in the newborn than in the adult. This difference in volume of distribution may be due to alteration in overall hepatic excretory function. The newborn excreted the taurocholate acid into the gut at a much slower rate than did the adult rats. This difference does not appear to be the result of the difference in availability of taurocholate to the transport system since the concentration of taurocholate in the liver of the newborn is as high or higher than that of the adult. Thus it has been concluded that the system that transports bile acids from the liver is not mature in the newborn rat and is probably largely responsible for the delayed disappearance of taurocholic acid from the plasma. It is difficult to determine from the present experiments if the transport system responsible for transfer of taurocholate from the plasma to the liver operates at the same capacity in the newborn as the adult; however, the liver/plasma ratio of taurocholate is similar in the two groups.

While it appears that the major reason for the delayed disappearance of taurocholic acid from the plasma of the newborn rat is due to a lower capacity of the liver to transfer taurocholic acid from the liver into the bile, the major defect in the liver of the newborn to handle ouabain (5, 8), BSP, and ICG (6) is the inability to transfer the xenobiotic from the plasma into the liver. Both ouabain (9) and taurocholic acid (10) are transported into the liver by an active carrier-mediated system. With ouabain there appears to be a complete inability of the liver of the newborn rat to concentrate it, while the liver of the adult rat can concentrate it about 30-fold over the level in the plasma (5). Taurocholic acid is also more concentrated in the liver than in the plasma but only about five times more than in the plasma. In contrast to the results observed with ouabain, the liver of the newborn rat can concentrate taurocholic acid to the same extent as that seen in the adult. This would suggest that taurocholic acid is taken up by a process other than the one that transports ouabain. Thus it would appear that there are at least four processes in the liver responsible for the transfer of organic substances from the plasma into the liver; one for most organic anions, one for organic cations, one for neutral substances, and another for bile acids.

Summary. The plasma disappearance and biliary excretion of taurocholic acid was found to be slower in the newborn than the adult rat. This appears to be due largely to a less efficient transfer of taurocholic acid from the liver into the bile, while the liver/plasma concentration ratio of taurocholate in the newborn is similar to that in the

adult. Ouabain, in contrast, cannot be taken up and concentrated in the liver of the newborn rat. This suggests that taurocholic acid and ouabain are transported into the hepatocyte by different systems.

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Influence of Cystic Fibrotic and Heterozygous Serum on Rat Jejunum[1] (39993)

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While the sweat test provides a highly reproducible and reliable test for the identification of the homozygous state of cystic fibrosis, assays which have been utilized for the identification of heterozygotes have provided less consistent results. Neither the rabbit trachea nor oyster gill assay have proven reliable when carried out under blind study conditions (1). The observation of Mangos and associates (2) that mixed saliva from homozygotes inhibited sodium reabsorption in the rat parotid gland suggested to Araki and associates (3) that cystic fibrotic plasma may contain a factor which inhibits sodium transport. Utilizing the rat jejunum, they observed that plasma from both homozygotes and heterozygotes, when added to the serosal surface just before mounting the tissue, inhibited short circuit whereas plasma from normals had no such effect. In a subsequent short communication Araki and associates (4) modified their technique in that the baseline short circuit current was recorded for 10 min, and the mucosal surface solution was then replaced by serum. Using this technique they found that sera from 48 of 50 cystic fibrosis patients and 40 of 46 heterozygotes decreased short circuit current. Two of twenty healthy controls showed false positives. The present study was undertaken to further study the reliability of the short circuit current method for distinguishing among cystic fibrotic, heterozygous, and normal sera.

Method. Short circuit current (Isc) and potential difference (PD) across the rat jejunum were determined according to the technique of Ussing and Zerahn (5). PD was measured using Ringer–3% agar bridges and calomel half-cells and monitored continuously employing a differential amplifier (Tetronics Model 3A9; input impedance, 1 MΩ) and displayed on an oscilloscope (Tetronics Model 561B). The output of the latter was recorded on a strip-chart recorder, and the calibration was checked using a Bioelectric Type CA5 calibrator. Current was applied through Ag-AgCl pellet electrodes and Ringer-agar bridges. Isc was determined using an HP 412A VTVM (internal shunt resistance <1 Ω), and the output was recorded on the strip recorder. Direct current resistance was calculated from PD/Isc.

The tissue was prepared as follows: Male Sprague-Dawley rats were anesthetized with pentobarbital sodium (60 mg/kg) ip, an abdominal incision was made, the jejunum was opened, and its lumen was washed out with bicarbonate-Ringer's solution henceforth referred to as Ringer's solution. The blood vessels to the jejunum were clamped with a hemostat, and a section was removed at a point usually 12–18 cm below the pyloric sphincter and placed in warm Ringer's solution. The tissue was cut longitudinally and mounted between two halves of a Lucite chamber which was then filled with Ringer's solution and oxygenated with a gas lift. The Lucite chamber was maintained at 37° using a warm-water jacket. Three minutes elapsed between clamping the blood supply to the jejunum and mounting the tissue. Sera were obtained from 10 normal, 17 cystic fibrotic, and 8 heterozygotic individuals and stored at 4° until testing which occurred at no later than 48 hr. Samples were assayed under blind study conditions after they were diluted by adding 2 parts of Ringer's solution to 1 part serum.

The protocol for assay was as follows: The rat jejunum was mounted, the tissue was allowed to stabilize for 10–15 min as indicated by the stability of the PD, and the control Isc was determined. The Ringer's solution on the mucosal side of chamber was then replaced with the diluted sera,

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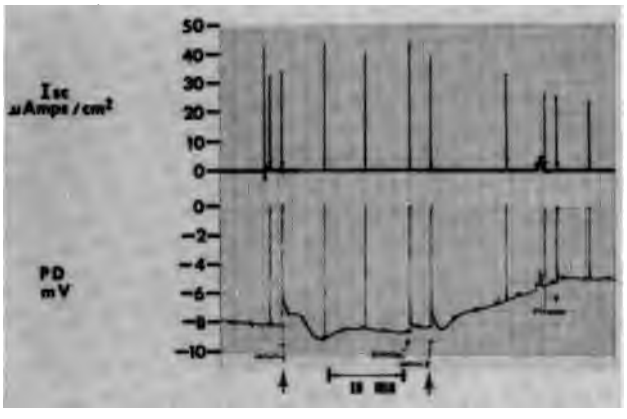
ording of the PD and determination Isc were continued for 15–20 min. sults were analyzed using a one-way s of variance and Scheffe's multiple ison test (6).

lts. Figure 1 is an original tracing g the influence of normal and CF on PD and Isc of the rat jejunum. pid upward deflections on the PD indicate the times at which the cur- s applied. In this experiment, serum arrow) was from a normal patient um 8 (second arrow) from a patient stic fibrosis. When the Ringer's so- on the mucosal surface was replaced rmal serum, a slight decrease in PD d, followed by an increase. This pat- as usually seen regardless of the source of the serum. The normal serum was replaced by Ringer's solution, the tissue was allowed to stabilize, and the Ringer's solution was then replaced by CF serum. The latter caused an initial decrease then increase in PD, followed by a more prolonged decrease.

Figure 2 is from an experiment in which the first unknown sample (arrow) that replaced the Ringer' solution was from a CF patient. Note the very substantial reduction in PD and Isc.

Figure 3 shows the influence of normal serum (first arrow) and serum from a heterozygous patient (second arrow) on PD and Isc. The experimental sequence was the same as that illustrated in Fig. 1. Note that the heterozygous serum had qualitatively



. The influence of normal (serum 9) and cystic fibrotic serum (serum 8) on the Isc and PD of the rat

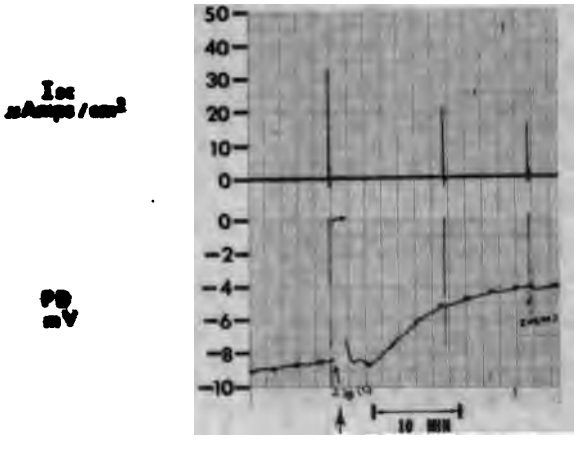


FIG. 2. Influence of cystic fibrotic serum (S.20) on the Isc and PD of the rat jejunum.

the same effect as serum from CF patients (Figs. 1 and 2). Figure 4 is from an experiment in which the first unknown sample (arrow) that replaced the Ringer's solution was from a heterozygote.

Before the replacement of the mucosal Ringer's solution with any sera from the 35 tissues studied, the mean potential difference was 7.44 ± 0.28 mV, the mean Isc was 37.6 ± 1.65 μA/cm^2, and the mean resistance was 203.9 ± 3.57 $\mu\Omega$/cm^2.

Figure 5 shows the individual changes which occurred in PD and Isc as a result of replacing the mucosal Ringer's solution with the various sera. Note that there is no overlapping between the normal serum and serum from either the CF or heterozygous patients. The mean change (±SE) in PD caused by normal serum was +0.29 ± 0.52 mV; for heterozygous serum it was −1.5 ± 0.31 mV, and for CF serum −2.38 ± 1.16 mV. With respect to Isc, normal serum increased it by 2.6 ± 4.5, heterozygous serum decreased it by 8.75 ± 3.58, and CF serum decreased it by 13.82 ± 5.84 μA/cm^2. The changes in the heterozygous and CF groups are significantly different from those of the normal group, but are not significantly different from each other. Thus while these experiments did not distinguish between CF and heterozygous serum, they did distinguish between normals and these two groups. The changes in PD and Isc were in general proportional so that none of the sera produced significant changes in membrane resistance.

Discussion. The results of the present experiments confirm the finding of Araki

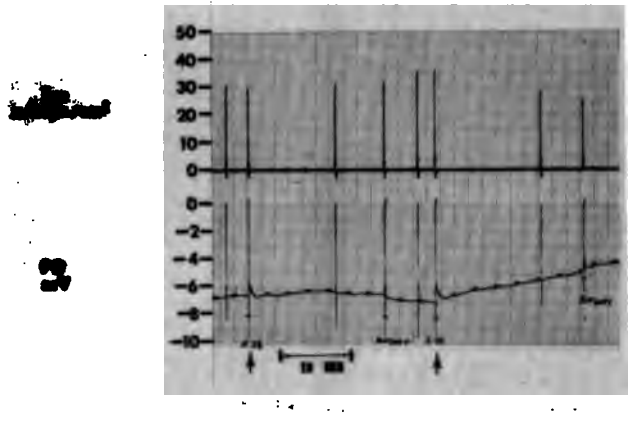


FIG. 3. Influence of normal (S.28) and heterozygous serum (S.21) on the Isc and PD of the rat jejunum.

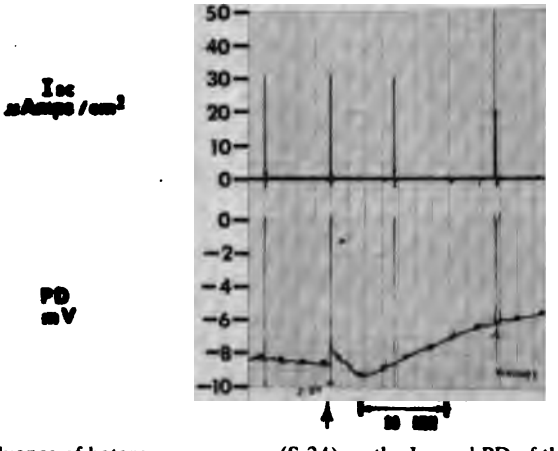
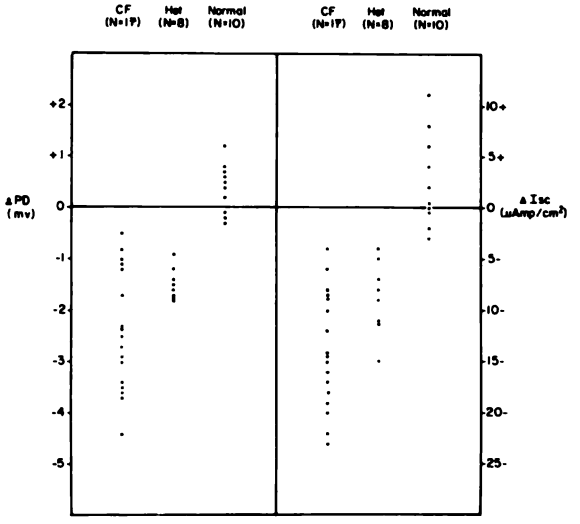


FIG. 4. The influence of heterozygous serum (S.24) on the Isc and PD of the rat jejunum.



Individual experiments showing the influence of the various sera on the PD and Isc of the rat

ociates (4) that both CF and hetero- sera decrease the PD and Isc of rat ı, while normal serum has no con- effect. Our failure to show false s or negatives may be due to the t the number of samples analyzed in ly was less than in that of Araki and es. However, even with our small number we found a clear separation n normal serum and CF and hetero- sera.

mechanism whereby CF and hetero- sera inhibit PD and Isc in the rat n is not known. However, it would that it in some way decreases the of sodium into the cell rather than a direct effect on a sodium-pumping ism. This idea is consistent with the of Kaiser and associates (7) that the n cystic fibrosis acts on the luminal the sweat gland but not on the uminal side and with our preliminary that CF serum has no effect on PD of the rat jejunum when placed on osal side. In both the sweat gland junum the passage of Na^+ into the l cell can be assumed to be with, e extrusion out of the serosal or uminal surface against, its electro- al gradient via a "pump" mechanism. actor in serum of either CF patients ıozygotes acted by inhibiting a so- dium pump one would expect an effect when applied to the serosal or contraluminal surface.

The factor involved in CF does not affect Na transport in all tissues. Its inhibitory effect on the eccrine sweat and salivary gland is not found in other tissues. In addition, in preliminary experiments we found no effect of CF serum on the PD or Isc of frog skin whether it was applied to the mucosal or serosal surface.

Summary. The rat jejunum bioassay has been shown to be capable of identifying both the cystic fibrotic homozygote and heterozygote and therefore would appear to have great potential as a screening test for the latter. It is suggested that the cystic fibrosis factor does not influence sodium reabsorption by inhibiting an active sodium reabsorption mechanism but rather by inhibiting passive sodium entry into the cell.

Dr. Kashinath D. Patil generously aided in the statistical analysis of the data.

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:s of Rheumatoid Factor and Complement on Soluble Immunoglobulin G Complexes: $^{51}CrCl_3$ Labeling and Precipitation Studies[1] (39994)

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ction. Soluble immunoglobulin G s which react with macromolecular id factor have been detected in ial fluid of patients with rheumaritis (1). This reaction has been ɔ accentuate the complement dim- the synovial fluid of these patients :nt studies (2, 3) have suggested ugh insoluble complexes are capaɔducing functional and metabolic ı normal neutrophils, soluble com- : not. These studies (2, 3) utilized id factor to convert soluble com- o insoluble complexes capable of ıg the biologic activity of normal ls. The present studies explore the rheumatoid factor and comple- he precipitation of $^{51}CrCl_3$-labeled beled soluble immunoglobulin G s.

ls and methods. Human immuno- G, obtained commercially (U.S. cal Corp., Cleveland, Ohio) as hun Fraction II, was solubilized in Cl (pH, 7.4). This solution conmunoglobulin G, immunoglobulin immunoglobulin A in a ratio of s determined by radial immunodif- Behring, Marburg, Germany). ssage through a Sephadex G-200 2.6 × 60.0-cm column at 25°), the total immunoglobulin G was a fraction corresponding to a moeight of approximately 150,000; nder was eluted as the macromoaction (void volume). A 1 g/dl ɔf this human immunoglobulin G was labeled with approximately 22.6 μCi of $^{51}CrCl_3$ (New England Nuclear, Boston, Mass., specfic activity approximately 375 Ci/g of Cr) per milliliter of immunoglobulin G at pH 7.4. After incubating for 1 hr at room temperature, the solution was passed over a Sephadex G-10 column (1.2 × 30.0 cm) to remove any unbound chromium. Tubes containing the maximum amounts of radioactivity and protein were pooled and subsequently heated at 63° for 7 min. After returning to room temperature, the solution was centrifuged at 10,000*g* for 15 min to remove any insoluble precipitate formed during the heating. The resulting slightly opalescent supernatant, upon passage over a Sephadex G-200 column, was found to contain a mean of 47.9% (range 37-60.5%) soluble immunoglobulin G complexes with the remainder of the protein in the form of 150,000 molecular weight immunoglobulin G. This solution was diluted with 0.9% NaCl to contain 2.4 mg/ml of soluble immunoglobulin G complexes and a mean of 1.13×10^5 cpm/mg of protein.

Rheumatoid serum was obtained with informed consent from patients with definite or classical rheumatoid arthritis and normal serum from healthy volunteers. The sera were either used immediately or frozen at −70° for use in future experiments. Rheumatoid factor titers were determined utilizing the commercial latex globulin reagent, Rheuma Quik (Biological Corp. of America, Port Reading, N.J.). All sera were spun at 10,000*g* for 15 min to remove any precipitate prior to use in the test system. In some studies, complement activity was removed from serum either by heat-inactivation for 30 min at 56° or by treatment with cobra venom factor (Cordis Corp., Miami, Fla.) for 30 min prior to use.

Complex reaction mixtures were prepared in a ratio of 1:2:5 by volume with

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soluble immunoglobulin G, normal serum, and rheumatoid serum, respectively. In studies using $^{51}CrCl_3$, these sera were previously treated with 0.05 ml of 1.3×10^{-3} *M* $CrCl_3$/5 ml of serum. Rheumatoid serum was utilized undiluted (Figs. 1 and 2) or was diluted with 0.9% NaCl to produce experimental sera with titers shown in Fig. 3. When either the rheumatoid or the normal serum was omitted from the mixture (Fig. 4), an equal volume of 0.9% NaCl was substituted to keep the reaction volume constant. After exposing the immunoglobulin G to 0.9% NaCl, rheumatoid serum, and/or normal serum, the respective tubes were centrifuged at 10,000*g* and washed twice in 0.9% NaCl. The supernates were discarded and the precipitates assayed in duplicate for radioactivity in a Nuclear Chicago gamma scintillation spectrometer. The precipitates were then resuspended in saline or phosphate-buffered saline and protein determinations were performed using the technique of Lowry *et al.* (4).

All data were statistically analyzed using Student's *t* distribution, since sample sizes were comparatively small in most of the experiments. Two-tailed Student's *t* tests were performed on all groups, and differences between these groups were considered to reach statistical significance when values of $P < 0.05$ were obtained.

Results. Figure 1 shows the effects of utilizing sera with increasing rheumatoid factor titers on the precipitation of ^{51}Cr-tagged soluble immunoglobulin G complexes. Although considerable variation occurred at each titer, differences in precipitation between negative sera and those containing rheumatoid factor became significant ($P < 0.05$) at titers of 1:80, were highly significant ($P < 0.001$) at titers of 1:160, and remained significant throughout the study to titers of 1:5120. Maximum precipitation apparently occurred in the region of rheumatoid factor titers of 1:1280, but there were not statistically significant

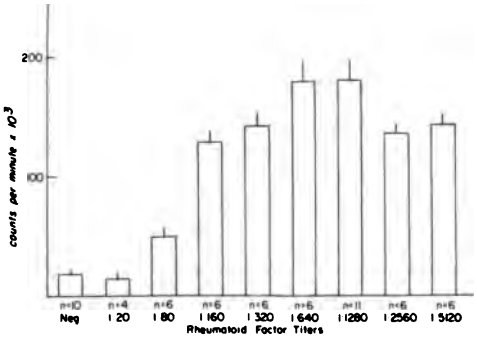

FIG. 1. Rheumatoid factor effects on soluble immunoglobulin G complex precipitation, $^{51}CrCl_3$ studies. Vertical lines on top of the bars = $\bar{X}$ + SEM. Differences between negative sera and those with rheumatoid factor become significant at titers of 1:80. Differences are not significant between any of the groups of sera with titers from 1:320 to 1:5120.

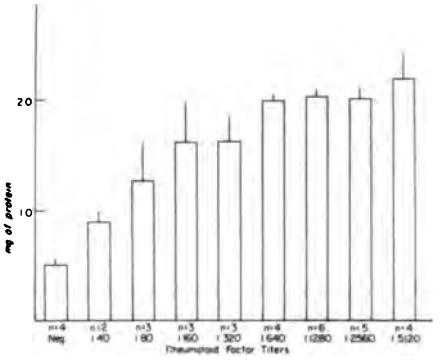

FIG. Rheumatoid factor effects on soluble immunoglobulin G complex precipitation, protein studies. Vertical lines on top of the bars = $\bar{X}$ + SEM. Differences between negative sera and those with rheumatoid factor become significant at titers of 1:160 and do not change significantly to titers of 1:5120.

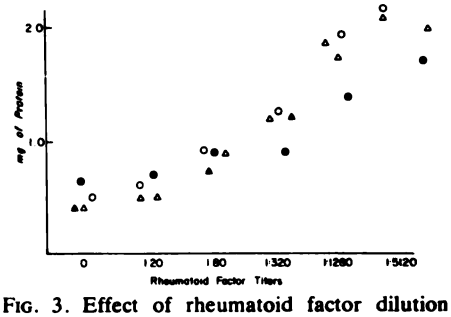

FIG. 3. Effect of rheumatoid factor dilution on soluble complex precipitation. ▲, ● = Two patients with initial rheumatoid factor titers of 1:5120; △,○ = 2 patients with initial rheumatoid factor titers of 1:2560; each symbol = $\bar{X}$ of four determinations. Differences between control and rheumatoid factor-containing sera become significant in the 1:80–320 range and there are no significant differences in precipitation from sera with titers from 1:1280 to 1:5120.

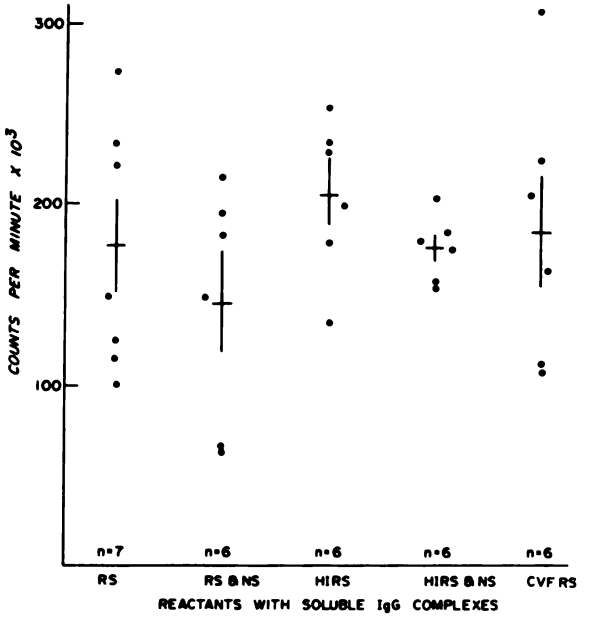

FIG. 4. Complement effects on soluble immunoglobulin G complex precipitation with rheumatoid factor. Crosses = $\bar{X}$ ± SEM, RS = rheumatoid serum, NS = normal serum, HI = heat-inactivated, CVF = cobra venum factor-treated. Treatments designed to decrease complement levels do not affect the ability of RS to precipitate ^{51}Cr-tagged soluble complexes.

differences between any of the sera groups exhibiting titers of 1:320 or above. Figure 2 shows similar results when nonradioactive soluble immunoglobulin G complexes are incubated with sera exhibiting rheumatoid factor titers up to 1:5120. Differences between negative and rheumatoid factor-containing sera became significant ($P < 0.05$) at titers of 1:160 and there were no statistically significant differences between any of the sera groups exhibiting titers of 1:160 or above. As shown in Fig. 3, serial dilutions of high-titered sera produced similar results. Differences between control and rheumatoid factor-containing sera became significant ($P < 0.05$) at titers of 1:80 (two patients) or 1:320 (two patients) and there were no statistically significant differences among any of the experimental sera exhibiting titers of 1:1280 or above. Figure 4 illustrates the precipitation obtained utilizing radioactively tagged soluble immunoglobulin G complexes and rheumatoid sera exhibiting a titer of 1:1280 but subjected to various treatments prior to incubation with the soluble complexes. As shown in this figure, there is no difference in the amount of precipitation from the soluble complex solutions whether rheumatoid serum (RS) is utilized alone, in the presence of normal sera (RS & NS), in the presence of rheumatoid factor sera which is heat-inactivated at 56° (HIRS), or in the presence of rheumatoid factor sera previously treated with cobra venum factor (CVFRS).

Discussion. $^{51}CrCl_3$ preferentially tags serum proteins (5) and has been utilized for *in vivo* studies of gastrointestinal protein loss (6). Previous studies (7) have shown that rheumatoid factor can cause specific precipitation when reacted with heat-aggregated γ-globulin but not with native immunoglobulin G. Previous precipitation studies (8, 9) have shown an inhibitory effect of native immunoglobulin G on soluble complex precipitation. Our studies utilize soluble complexes in equal volumes to native immunoglobulin G, and demonstrate increasing precipitation of the complexes from solutions incubated with rheumatoid sera to rheumatoid factor titers of 1:320 and 1:160 utilizing chromium-tagged or untagged complexes. A similar increase in precipitation with increasing amounts of rheumatoid fac-

tor to titers of 1:1280 is demonstrated in serial dilution studies of experimental sera and has been reported (10) in previous studies utilizing different methods. Our studies confirm these earlier findings and demonstrate that the chromium-tagged system yields a precipitin curve similar to that seen when nonradioactive soluble complexes are utilized. These studies suggest that increasing serum concentrations of rheumatoid factor, up to titers of 1:1280, yield increasing amounts of precipitate when exposed to a constant amount of soluble immunoglobulin G complexes in the presence of native γ-globulin.

Although rheumatoid factor alone (11) and rheumatoid factor-immunoglobulin G complexes have been shown to fix complemen (9), our studies utilizing ^{51}Cr-labeled complexes and high-titer (1:1280) rheumatoid sera confirm previous reports (12) suggesting that complement is not required for maximum precipitation of soluble complexes by high-titer rheumatoid factor-containing serum. This study has thus shown that a chromium tag applied to soluble immunoglobulin G complexes can be used to determine quantitatively the precipitation of these complexes. The measurable precipitate increases proportionately with increasing titers of rheumatoid factor up to 1:320 and is not affected by measures designed to eliminate complement from high-titer rheumatoid sera. These studies confirm previous suggestions that increasing titers of rheumatoid factor may increase the biologic activity of immunoglobulin G complexes, and demonstrate the usefulness of a ^{51}Cr tag in delineating the interaction of these complexes with rheumatoid factor.

Summary. $^{51}CrCl_3$-labeled soluble immunoglobulin G complexes have been shown to have similar precipitation characteristics to unlabeled complexes in the presence of increasing amounts of macromolecular rheumatoid factor. Complement depletion of high-titer rheumatoid sera did not appear to influence these interactions which may be important components of rheumatoid inflammation.

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and Serological Evaluation of a Meningococcal Polysaccharide Vaccine Groups A, C, and Y (39995)

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purified capsular polysaccharide epared against groups A, C, and *a meningitidis* have been devel-). These vaccines have been e antigenic and free of significant s (3-6). Subsequently, group A, nd group A/C combined vaccines licensed for limited distribution the Bureau of Biologics, Food Administration.

to broaden the potential protec-ed by a meningococcal vaccine, Y polysaccharide component was roups A and C polysaccharides. se of this study was to evaluate ibination vaccine with respect to ceptability and serological re-

and methods. Vaccines. The ed in this study (Lot 2501) was n the Merrell-National Research es using *Neisseria meningitidis* A-nd 6306 Y (Slaterus Y) obtained Walter Reed Army Institute of The cold phenol method of *et al.* (7) was used to purify the rides. Molecular sizing of the po-es using Sepharose 4B gel per-romatography suggested that the ponents of the vaccine were of ular weight. The partition coeffi-) at the Ve were 0.26, 0.22, and ectively, for the A, C, and Y rides. The individual polysaccha-nents, A, C, and Y, were tested nicity (three rabbits each compo-25 μg/kg. The mean temperature 0.2, 0.2, and 0.0° for the three components, respectively. The final vaccine was tested in eight rabbits at a concentration of 0.075 μg/kg (0.025 μg each component) with an average rise of 0.39°. These results are within limits established for meningococcal vaccines (8, 9). In addition, the vaccine met the general requirements of the Bureau of Biologics, Food and Drug Administration (8), and other existing standards pertinent to the use of vaccines (9).

A placebo consisting of isotonic sodium chloride with 0.01% thimerosal was used in this study.

Clinical studies. The study population consisted of healthy adult volunteers, employees and staff of a university hospital, who had given their informed consent. Both men and women, 18 to 57 years of age (mean age, 25.4 years), were included in the study population. The vaccine was administered to 30 individuals, each of whom received 0.5 ml (containing 50 μg of each of the three polysaccharides) subcutaneously, in the upper arm in the region of the deltoid muscle. Twenty-three of these individuals had not been immunized with a meningococcal vaccine. Of the other seven individuals, four had been immunized with a group A/C combined vaccine, two had received groups A and C vaccines, and one had been given a group C vaccine. All previous immunizations had been with 50 μg each of the respective polysaccharides. Five individuals received 0.5 ml of the placebo.

Blood specimens were obtained by venipuncture (antecubital vein) prior to immunization and 3 weeks later. Sera were collected and stored frozen at −20° until serological testing was conducted.

Clinical observations were made and recorded for each individual at 4-hr intervals

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until bedtime on the day of immunization, then daily for the next 3 weeks. Oral temperatures were taken for the first 3 days regularly, and continued if the volunteer had any symptoms. Any adverse local or systemic reactions were recorded on individual case report forms.

Bactericidal tests were performed on pre- and postimmunization sera (inactivated at 56° for 30 min) using a procedure developed by the Bureau of Biologics (8).

Results. Of the 30 individuals who received the vaccine, 12 reported symptoms of a systemic nature. Temperatures above 37.2° were observed in 6 individuals. In 5 of these 6 volunteers maximum temperatures ranged from 37.3 to 38.8°. Other temperature readings and symptoms further suggested a mild systemic reaction to the vaccine. Other symptoms of these 5 individuals were recorded on days postimmunization as follows: 1 individual, Day 1; 1 individual, Days 1 and 2; 2 individuals, Days 2 and 3; and 1 individual, Day 3. The sixth individual was reported to have had a temperature of 39.4° four hours after immunization, however, no other temperatures were abnormal and no other adverse reactions were observed. Complaints recorded for all 12 individuals above were headache (six instances), coryza (three instances), lethargy (three instances), nausea (three instances), and chills (one instance). All symptoms dissipated by Day 3.

Twenty-three of the 30 vaccinees (one of five placebos) complained of local pain or soreness at the site of injection, with the discomfort lasting 1 day for 12 individuals, 2 days for 4 individuals, and 3 days for 7 individuals.

No correlation was noted between symptoms observed and prior immunization with meningococcal vaccines.

Results of bactericidal antibody tests (Table I) of the sera from the 23 individuals not previously immunized with a meningococcal vaccine indicated that 23 (100%), 21 (91.3%), and 20 (87.0%) had significant (fourfold or greater) increases in group A, group C, and group Y antibodies. If individuals with high preexisting antibody levels are excluded, 95% or more of the individuals developed significant antibody levels to all three vaccine components.

The serological responses of seven individuals, after receiving the ACY vaccine, who had received meningococcal vaccine A, C, or A/C, are shown in Table II. All individuals developed high levels of group Y bactericidal antibodies. One individual who had previously received group C vaccine responded to the group A and group Y polysaccharides. Four of six individuals had serological rises of group A antibodies after their second contact with this polysaccharide. None of the seven individuals had any rises to group C antibodies, suggesting no booster effect with this particular polysaccharide.

Discussion. This study indicates that the

TABLE I. SERUM BACTERICIDAL ANTIBODY RESPONSES IN HUMAN SUBJECTS[a] MEASURED 3 WEEKS AFTER A SINGLE DOSE OF MENINGOCOCCAL POLYSACCHARIDE VACCINE, GROUPS A, C, Y.[b]

	Initial seronegatives			Initial seropositives		
Vaccine component	≧4 × Rise, No./total[c]	%	GM[d]	≧4 × Rise, No./total	%	GM
			<4			45
Group A	22/22	100	226	1/1	100	181
			<4			20
Group C	21/22	95	99	0/1	0	20
			<4			333
Group Y	19/20	95	333	1/3	33	1024

[a] Twenty-three individuals, no previous meningococcal vaccines.
[b] Fifty micrograms of each polysaccharide.
[c] Number showing rise/total number tested.
[d] Geometric mean titer.

TABLE II. RESPONSE OF VACCINEES TO GROUPS A. C, Y MENINGOCOCCAL VACCINE BY INDIVIDUALS PREVIOUSLY IMMUNIZED WITH GROUPS A AND/OR C VACCINES.

Individual	Previous meningococcal vaccine[a]/response[b]	Interval (months) before ACY vaccine	Response to ACY components[b]		
			A	C	Y
1	C+	26	+	−	+
2	A−, C+	27	+	−	+
3	A+, C+	27	+	−	+
4	A+/C+	13	−	−	+
5	A+/C+	31	+	−	+
6	A−/C−	19	−	−	+
7	A+/C+	19	+	−	+

A, C, A/C denotes group A, group C, groups A/C combined meningococcal vaccines.
Response + = fourfold or greater rise in bactericidal antibody.

ıups A, C, and Y polysaccharide vaccine, ile causing some mild reactions, is safe l clinically acceptable.

The serological conversions induced as a ult of the immunization are of particular ɛrest. A high percentage of those individ- s with no prior experience with menigo- ːcal vaccines developed significant levels bactericidal antibodies to all three com- ıents of the vaccine. This suggests an ependent immune response to each pol- ccharide. The mean antibody level to group Y polysaccharide component was : different from that observed with mon- ılent group Y vaccines, 333 versus 359 . In another clinical study in our labora- y with group C vaccine, the mean bacte- ıdal antibody titer was 85, which was not ferent from the mean group C antibody ːr observed in this study. In a separate ıical study with a new group A vaccine, : mean bactericidal antibody level was 3. Other clinical studies with A/C com- ıed vaccine resulted in mean bactericidal ːibody levels to the group A component 695 and to the group C componènt of 8.

Further evidence of the independent re- ɔnse to meningococcal polysaccharide s the observation that individuals with or experience with group A and group C lysaccharides developed bactericidal an- odies to the group Y component of the ːY vaccine.

A suggestion of a booster effect was ob- ved with the group A component, but : the group C component of the ACY ːcine. A correlation has been made be- ːen the presence of bactericidal antibod- ies and resistance to meningococcal disease (10). Since clinical efficacy has been shown with polysaccharide vaccines of group A and C meningococci (4–6) which induce antibody levels comparable to those observed in this study, it seems probable that the groups ACY polysaccharide vaccine would be clinically efficacious.

Summary. The clinical and serological testing of a groups A, C, and Y meningococcal polysaccharide vaccine is described. Some minor reactions were observed. High levels of bactericidal antibody to all three components were observed in 90% of the individuals 3 weeks after receiving the vaccine.

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ıibition of Interferon Induction in Mice by Mycoplasmas and Mycoplasmal Fractions (39996)

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ngle injection or repeated injections interferon inducer may result in a ɔf hyporeactivity in which the host ːes progressively less interferon with ıccessive challenge (15, 16, 22, 28). ıenomenon has limited the usefulness ːrferon inducers in the treatment of isease.

ːnt studies in our laboratories have that a variety of mycoplasmas in- pH-stable interferon following in- on of sheep and human leukocyte ːs *in vitro* and that the lymphocyte ːs to be the active interferon-produc- ll. Other investigations have shown ycoplasmas can also induce interferon ɔ in mice. Further studies demon- that mice became hyporeactive to ron induction by NDV or poly(I:C) noculation with viable *Mycoplasma dis* (5). This mycoplasma species in- an acute arthritis in rats (9) and a ː arthritis in mice (7). It has also eported to be immunosuppressive *in* ', 17) as well as inhibiting lymphocyte ; *in vitro* (1, 20, 26).

present investigation was undertaken ːrmine whether mycoplasma-induced ːactivity was specific for *M. arthritidis* further characterize the mycoplasma nent responsible.

ːrials and methods. *Cultivation of my- nas. Mycoplasma arthritidis* strain p10 (12) and *M. pulmonis* JB, des- ɪ "TR" (obtained from D-Taylor- ɜon), were grown on mycoplasma r in broth (Difco Laboratories, De- Mich.) supplemented to final concen- s of 15% (v/v) horse serum, 5% (v/ h yeast extract, and 1000 U of peni- ıl (3, 13). *Mycoplasma pulmonis* re- an additional supplement of 1% (v/v) of a 2 mg/ml solution of diphosphopyridine nucleotide (DPN-102, Sigma Chemical Co., St. Louis, Mo.). *Acholeplasma laidlawii* strain BNl-Nal^r was obtained from Dr. A. Liss (Storrs, Conn.) and strain 1305 was obtained from Dr. R. N. Gourlay (Compton, Surrey, England). Both acholeplasmas were cultured in tryptose broth supplemented with 1% (v/v) serum fraction, 1% (w/v) glucose, and 1000 U of penicillin/ml (19).

Mycoplasma pools were harvested by centrifugation, assayed for colony-forming units (CFU), and stored at −70° as previously described (12). Sonified mycoplasma suspensions were prepared by washing the organisms ×3 in phosphate-buffered saline (PBS), resuspending in deionized water, and exposing to 1-min periods of ultrasonic vibration using a Model S75 Branson sonifier until free of viable organisms. Dilutions of sonified suspensions were prepared in PBS.

Crude membranes were removed from sonified suspensions by centrifugation at 20,000 rpm using an SS34 rotor (Sorvall RC5 centrifuge). The membrane sediment was resuspended in PBS, washed ×3 by additional centrifugation, and finally resuspended to the volume of the original mycoplasma suspension. In experiments to compare the hyporeactive-inducing ability of whole organisms, sonified organisms, or membranes, all preparations were made from aliquots of the same mycoplasma suspension.

Virus cultivation. The Hertz strain of NDV (Courtesy of Dr. S. Baron, University of Texas, Galveston, Tex.) was grown in embryonated chicken eggs and titered 10^9 plaque-forming units (PFU) in primary chicken embryo cells (28).

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Mice. Seven- to eight-week-old female Swiss–Webster mice (Simonsen Laboratories, Gilroy, Calif.) were used throughout these studies.

Cell culture and interferon assays. Mouse interferon was assayed on mouse L cells (L929, obtained from the American Type Culture Collection, Rockville, Md.) grown in Eagles minimal essential medium (MEM) supplemented to final concentrations of 10% (v/v) fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The 50% plaque reduction technique employing vesicular stomatitis virus (VSV) as the challenge virus was used for interferon assays (21). An internal mouse interferon standard consisting of a known poly(I:C)-induced serum interferon was included with each set of assays. This internal standard was compared with an international reference mouse interferon obtained from the National Institutes of Health. The international interferon standard (designated titer of 6500 units) titered 6847 ± 2664 units in our assay system (10 determinations).

Demonstration of hyporeactivity in vivo. Appropriate dilutions of the mycoplasma suspensions were injected intraperitoneally (ip) in 0.2-ml amounts into groups of three mice each. Uninjected mice or mice injected with PBS or mycoplasma broth medium served as negative controls. For the demonstration of hyporeactivity the injected mice were challenged with 0.2 ml of NDV 1, 3, and 6 days later. Mice were exsanguinated 6 hr after inoculation of the virus, about the time of maximum interferon induction by NDV. The resulting sera from each group of three mice were pooled. In a previous study (5) we showed that inhibition of interferon induction by *M. arthritidis* was not caused by an alteration in the kinetics of induction by NDV but was in fact due to an actual inhibition of the response. As a positive control for hyporeactivity, mice were given a preliminary injection of NDV and were rechallenged with NDV 1, 3, and 6 days later. Prior to interferon assay, all serum samples were treated at pH 2.0 for 5 days at 4°.

Results. Inhibition of the interferon response by M. arthritidis. Lack of requirement for viable organisms and kinetics of the hyporeactive response. The ability of various concentrations of viable and nonviable, sonified *M. arthritidis* to induce hyporeactivity to interferon induction by NDV in mice was compared. The results are summarized in Table I. Viable mycoplasmas induced significant hyporeactivity at a concentration of 5×10^9 CFU per mouse 1 and 3 days postinoculation (200 and 630 units of interferon, in comparison with control values of 4800 and 3200 units, respectively). In contrast, a dose of 5×10^8 CFU per mouse induced only minimal hyporeactivity at 1 day, and 5×10^7 CFU per mouse did not result in any detectable inhibition of the interferon response to NDV. In this experiment, nonviable sonified preparations of the same *M. arthritidis* pool induced a more striking hyporeactive effect than did the viable preparations. Thus, a complete inhibition of the interferon response (<50 units of interferon) was apparent 1 day after injection of 5×10^7 sonified organisms, and a suppressed interferon response was still apparent by 3 days. A dose of 2×10^{10} disrupted organisms per mouse virtually eliminated all interferon production through 6 days (data not shown).

Additional experiments were conducted to determine how soon after the injection of *M. arthritidis* the hyporeactive state developed. Mice were injected ip with 10^9 nonviable sonified or 5×10^9 viable suspen-

TABLE I. INHIBITION OF THE INTERFERON RESPONSE OF MICE TO NDV FOLLOWING INOCULATION WITH VIABLE AND NONVIABLE *M. arthritidis*.

First injection	Interferon response[a] of mice to NDV challenge, Days after first injection		
	1	3	6
M. arthritidis			
5×10^7 CFU, viable	4200	5500	4300
5×10^8 CFU, viable	1400	3200	3200
5×10^9 CFU, viable	200	630	3200
5×10^7 CFU, sonified[b]	<50	800	3200
5×10^8 CFU, sonified	200	50	3200
5×10^9 CFU sonified	<50	<50	800
NDV, 10^8 PFU	100	<50	1300
Mycoplasma broth	4800	3200	3500

[a] Interferon titers measured 6 hr post-NDV injection.

[b] Mycoplasma counts performed prior to sonification.

f *M. arthritidis* in PBS. Mice injected BS served as negative controls for activity. At 2, 6, 14, or 24 hr, mice hallenged with NDV and serum was ed 6 hr later for interferon assays. ıtrol for interferon induction by the lasmas themselves, additional mice led without NDV challenge 8 and 12 : injection of the mycoplasmas. These epresent the peak of the interferon se of mice to mycoplasmas (24). The are summarized in Table II. Sonified lasmas did not induce a detectable ron response in mice 8 or 12 hr ection. The 200 units of interferon d in mice by viable mycoplasmas 8 tinjection was insufficient to signifi- alter the interferon titers resulting NDV challenge at the 2- and 6-hr eriods. Thus there was no evidence oreactivity in mice 2 or 6 hr postin- on with sonified or viable mycoplas- ı contrast, mice injected 14 or 24 hr ısly with *M. arthritidis* exhibited a d suppression of the interferon re- to NDV challenge.

oreactivity induced by M. pulmonis laidlawii. Since mycoplasmas exert ty of diverse effects on cell functions and *in vivo*, experiments were next ted to determine whether the ob- suppression of interferon induction *arthritidis* could also be obtained by species of mycoplasma. In the first nent, mice received primary injec- of (i) mycoplasma broth, (ii) NDV, . *pulmonis* JB (1.8×10^9 CFU), or . *pulmonis* JB (1.8×10^8 CFU). cted mice served as controls. After r 6 days, all animals were challenged IDV and were exsanguinated 6 hr The interferon levels obtained are summarized in Fig. 1. Mice receiving a primary injection of mycoplasma broth produced interferon levels in response to challenge with NDV similar to those seen in uninjected mice challenged with NDV. Mice injected with NDV produced <50 units of interferon when challenged with NDV 1 and 3 days later, and produced only 150 units of interferon when challenged 6 days later with NDV. Mice receiving 1.8×10^9 CFU per mouse of *M. pulmonis* exhibited a marked hyporeactivity to NDV-induced interferon through 3 days, but appeared normal by 6 days. Decreasing the dose of *M. pulmonis* 10-fold resulted in no significant hyporeactivity. In a repeat experiment, a primary injection of *M. pulmonis* JB (2×10^9 CFU per mouse) decreased the interferon response to NDV from 1800 units of interferon in control

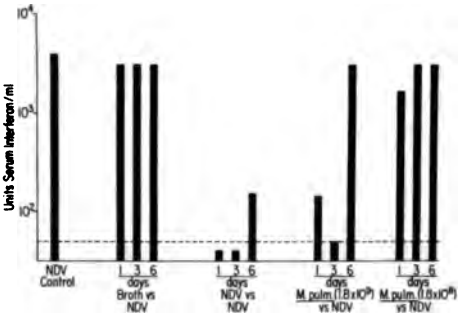

FIG. 1. Effect of *M. pulmonis* on the interferon response of mice to NDV. NDV control: Serum was collected 6 hr after the ip injection of NDV into normal mice. In the other groups, mice receiving a primary ip injection of mycoplasma broth, NDV, or 1.8×10^9 and 1.8×10^8 CFU of *M. pulmonis* strain JB were challenged ip 1, 3, and 6 days later with NDV. Serum for interferon assay was collected 6 hr post-NDV injection. The dotted line represents the level of sensitivity of the assay system, i.e., 50 U of interferon.

TABLE II. KINETICS OF THE HYPOREACTIVE RESPONSE.

	Interferon response to first injection after		Interferon response[a] to NDV challenge, Hours after first injection			
First injection	8 hr	12 hr	2	6	14	24
itidis, 10^9 CFU, sonified[b]	50	50	1900	5200	575	226
itidis 5×10^9 CFU, viable	200	NT[c]	8000	9500	90	200
			7500	7400	NT	2300

rferon titers measured 6 hr post-NDV injection, i.e., at 8, 12, 20, and 30 hr post-mycoplasma injection.
oplasma counts performed prior to sonification.
tested.

mice to 130 units after 1 day and 710 units after 3 days. By 6 days after injection of *M. pulmonis*, interferon levels increased in response to NDV, i.e., 980 units. Similar results were also obtained when *M. pulmonis* strain TR was tested for its ability to induce hyporeactivity.

Experiments were also conducted using *A. laidlawii* strain 1305 (Fig. 2). A primary injection of mice with tryptose broth (used for the culture of *A. laidlawii*) did not affect the ability of mice to produce interferon in response to a challenge injection of NDV. In contrast, primary injections of NDV and 5×10^9 CFU per mouse of *A. laidlawii* resulted in complete hyporeactivity through 3 days to interferon induction by a challenge injection of NDV. When approximately 10-fold fewer acholeplasmas were administered, hyporeactivity was complete at 24 hr, but only partial at 72 hr postinoculation. The interferon-producing capacity of the animals was normal 6 days postinoculation. The experiment was repeated with *A. laidlawii* strain BNl-Nal^r, using primary injections of 10^7 or 10^9 CFU/ml. The development of hyporeactivity followed a similar time sequence and was again shown to be dose dependent.

Further characterization of the mycoplasma component inducing hyporeactivity. In the next series of experiments viable, sonified, and membrane preparations of *A. laidlawii* Nal^r and *M. arthritidis* 14124 p10

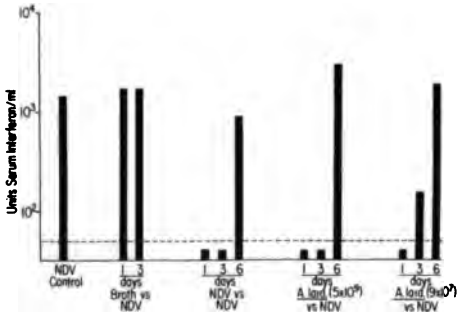
Units Serum Interferon/ml
NDV Control
1 3 days Broth vs NDV
1 3 6 days NDV vs NDV
1 3 6 days A laid (5x10⁹) vs NDV
1 3 6 days A laid (9x10⁷) vs NDV

FIG. 2. Effect of *A. laidlawii* on the interferon response of mice to NDV. NDV control: Serum was collected 6 hr after the ip injection of normal mice with NDV. In the other groups, mice receiving a primary ip injection of tryptose broth, NDV, or 5×10^9 and 9×10^7 *A. laidlawii* strain 1305 were challenged ip 1, 3, and 6 days later with NDV. Serum for interferon assay was collected 6 hr post-NDV injection.

were compared for their ability to suppress the interferon response of mice to NDV. The results are summarized in Table III. Both viable and sonified preparations of *A. laidlawii* and *M. arthritidis* resulted in a decreased interferon response to NDV. The crude membrane fractions prepared from *A. laidlawii* induced a striking hyporeactive effect through 6 days; those prepared from *M. arthritidis* induced hyporeactivity through 3 days.

To further characterize the component of mycoplasmas responsible for hyporeactivity, mice were injected ip with a sonified preparation of *M. arthritidis* 14124 p10 which had been heated to 56° for 1 hr or to 120° for 15 min. Mice injected with a sonified unheated preparation served as controls. The results are summarized in Table IV. In the first experiment, mice treated with unheated, sonified mycoplasmas were markedly hyporeactive to interferon induction in response to NDV through 3 days, whereas mice pretreated with heated material exhibited a normal interferon response to NDV at all time periods of challenge. This observation was confirmed in the second experiment since heat treatment of sonified *M. arthritidis* prior to injection ($5 \times$

TABLE III. DEVELOPMENT OF HYPOREACTIVITY TO INTERFERON INDUCTION IN MICE FOLLOWING THE INJECTION OF VIABLE, SONIFIED, AND CRUDE MEMBRANE PREPARATIONS OF VARIOUS MYCOPLASMAS.

First injection	Interferon response[a] to NDV challenge, Days after first injection		
	1	3	6
A. laidlawii Nal^r			
Viable, 1.6×10^9 CFU	<50	400	600
Sonified, 1.6×10^9 CFU[b]	<50	<50	200
Membranes from 1.6×10^9 CFU	80	50	70
M. arthritidis 14124 p10			
Viable, 1.6×10^9 CFU	<50	<50	550
Sonified, 1.6×10^9 CFU	200	90	3600
Membranes from 1.6×10^9 CFU	50	290	3200
Tryptose broth	3000	3200	NT[c]
Mycoplasma broth	1600	3200	NT

[a] Serum interferon levels were determined 6 hr post-NDV challenge.
[b] CFU prior to sonification.
[c] Not tested.

LE IV. EFFECT OF HEAT TREATMENT ON THE ABILITY OF THE HYPOREACTIVITY-INDUCING COMPONENT OF *M. arthritidis*.

	Interferon[a] response of mice to NDV challenge. Days after the first injection		
First injection	1	3	6
rthritidis, 8 × 10^8 U,[b] unheated	50	<50	1900
rthritidis, 8 × 10^8 U, 56° 1 hr	4500	6600	9000
rthritidis, 8 × 10^8 U, 120° 15 min	4100	7100	6400
	<50	<50	4500
	6300	NT[c]	NT
rthritidis, 5 × 10^9 U, unheated	<50	<50	800
rthritidis, 5 × 10^9 U, 56° 1 hr	1200	800	3200
rthritidis, 2 × 10^{10} U, unheated	<50	<50	70
rthritidis, 2 × 10^{10} U, 56° 1 hr	<50	<50	<50
, 10^8 PFU	100	<50	1300
plasma broth	4800	3200	3500

um interferon levels were determined 6 hr DV challenge.
U before sonification.
t tested.

FU per mouse) markedly decreased bility of the preparation to induce eactivity. However, hyporeactivity ot abolished by heating (56°, 1 hr) the initial injection of *M. arthritidis* creased to 2 × 10^{10} CFU per mouse.

cussion. The present studies have that three biochemically distinct my- ma species are capable of inhibiting terferon response of mice to NDV. oted mycoplasmas were also active in ng the hyporeactive state, and the lasma component responsible was abile and appeared to be associated iembranes.

mechanism of the mycoplasma-me- inhibition of the interferon response could be related to a general toxic on host functions since some myco- as, including membrane preparations, been reported to exhibit toxic proper- , 10, 11, 30). However, the ability of lasmas to induce interferon (8, 24) o the development of the hyporeactiv- ity state is not consistent with this hypothesis.

Since *M. arthritidis* is known to inhibit lymphocyte mitogenesis and antibody synthesis *in vitro* by arginine depletion of culture media (1, 20, 26), it might be argued that a similar mechanism is responsible for the immunosuppressive and interferon-inhibiting properties of *M. arthritidis in vivo* (2, 5, 17). In the latter regard it is relevant that only lymphocytes produce interferon in response to *in vitro* infection with mycoplasmas (4). However, significant arginine depletion *in vivo* is not a likely event. Our present findings that two glucose-utilizing mycoplasmas inhibit the interferon response *in vivo* indicates that another mechanism is operative.

Recent work by Stringfellow and Glasgow (29) has shown that serum taken from mice made hyporeactive to interferon production by injection with encephalomyocarditis virus (EMC) contains a factor which confers the hyporeactive state to mouse embryo fibroblasts *in vitro*. Furthermore, this factor was distinct from interferon and was produced at a time when the interferon response had abated (27). Preliminary experiments in our laboratories (unpublished data) indicate that serum taken from mice 24 to 72 hr postinoculation with *M. arthritidis* also contains a factor which inhibits the *in vitro* interferon response of mouse embryo fibroblasts to NDV. The physicochemical relationship of this factor to interferon and to the hyporeactive factor described by Stringfellow (27) is currently under study.

The finding that a variety of mycoplasmas can induce interferon and hyporeactivity to interferon induction *in vivo* clearly provides the potential for altered host susceptibility to infection with viruses. Thus an increased resistance to viral infection could occur during the early phase of mycoplasma infection in which serum interferon was present. In this regard, Fauconnier and Wroblewski (8) presented evidence that a primary injection of mice with an interferon-inducing acholeplasma species protected the animals against infection with Semliki Forest virus. In contrast, a decreased resistance to viral infection might occur during the hyporeactive state induced by mycoplasmas. It is of inter-

est that in certain circumstances mixed mycoplasma/virus infections of avian (14, 23) and porcine (18) hosts appear to result in more severe disease.

Summary. The injection of both viable and sonified nonviable preparations of *M. arthritidis*, *M. pulmonis*, and *A. laidlawii* into mice suppressed the interferon response of the animals to challenge with Newcastle disease virus (NDV). The inhibitory effect was dependent upon the numbers of organisms injected. The suppression of the interferon response was first apparent 14 hr postinjection and reached a maximum 1 and 3 days postinjection. High doses of organisms produced an inhibitory effect through 6 days. The mycoplasma component responsible for induction of hyporeactivity appeared to reside in the membrane fraction of the organisms. Heat treatment of mycoplasma preparations abolished the effect except at very high inocula.

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lation by Gastric Inhibitory Polypeptide of Insulin and Glucagon Secretion by Rat Islet Cultures[1] (39997)

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in secretion is greater when glucose ı orally compared to intravenously ıis has been attributed to the insu- ɔic effect of certain gastrointestinal ıes that are released by oral glucose. ly, evidence suggests that a leading ıte for this role is gastric inhibitory ɔtide (GIP), which is secreted after t not intravenous glucose (2, 3).

ulation of insulin release by GIP ad- ation has been shown *in vivo* (2, 4). *vitro* stimulatory effect of GIP on secretion by the isolated perfused ıcreas has been demonstrated at a ogic concentration of GIP (1 ng/ml), y high concentrations (1–10 μg/ml) ıecessary to elicit a modest rise in release by isolated rat islets (5, 6). :he collagenase method of isolating ıay alter the plasma membrane and fect the responsiveness of these cells, :stigated GIP action on cultured islet

rials and methods. Monolayer cul- ɔf the pancreas of 2- to 5-day-old rats were established as previously ed (7, 8). Four days following initia- the cultures, experiments were per- .

:riments were done in Earle's balanced salt solution (EBSS) (9) supplemented with bovine serum albumin (0.25%, w/v). A 2-hr preincubation interval was followed by a 1-hr incubation in the test medium, which consisted of EBSS with bovine serum albumin and varying amounts of glucose (0, 1.7, 5.5, 8.3, or 16.5 m*M*), with or without essential and nonessential amino acids (10), and with gradients of GIP (0, 1, 10, 100, or 1000 ng/ml; purchased from Dr. J. C. Brown). All incubations were performed at 37° in a humidified automatic CO_2 tissue culture incubator with an atmosphere of 95% air and 5% CO_2.

Immunoreactive insulin (IRI) and immunoreactive glucagon (IRG) were measured by cellulose radioimmunoassay (11, 12). Antiserum 30K was used to measure IRG. All samples for IRG were collected in benzamidine (final concentration, 0.05 *M*) to reduce hormone degradation, as previously reported (12). Results are expressed as mean microunits per milliliter (IRI) or mean picograms per milliliter (IRG) ± 1 SD. Significance was determined by two-tail Student's *t* test.

The binding of GIP to cells was assessed by an immunoperoxidase technique (13). Cultures were fixed in Bouin's solution for 2 hr, then sequentially incubated at 4° for 30 min each with GIP (10 ng/ml), rabbit anti-GIP serum, and horseradish peroxidase-labeled sheep anti-rabbit γ-globulin, and then incubated with 3,3′-diaminobenzidine (which served as substrate for the peroxidase reaction) and hydrogen peroxide for 45 min at room temperature. Control experiments were done in which one or more of the components needed was omitted, and these specimens were negative for

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staining. In addition, when rabbit anti-GIP was first incubated with GIP and then used, no staining occurred.

Results. At 16.5 m*M* glucose, a concentration of GIP as low as 1 ng/ml significantly enhanced IRI release (Table I). However, when glucose was 1.7 m*M*, GIP concentration of 10 ng/ml or greater were required to increase IRI release significantly. The relative stimulatory effect of GIP on IRI secretion was greater when glucose was elevated or when amino acids were absent. However, as shown in Table II, since the amino acid mixture itself enhanced glucose-stimulated IRI secretion, this may have obscured any potential additional stimulatory activity of GIP.

GIP at 1 ng/ml significantly increased IRG secretion (Table III). Since stimulation was seen even at glucose m*M* 16.5, this action apparently overrides any suppressive effect that high glucose levels may have. Since IRG secretion was enhanced by the presence of the amino acid mixture, when amino acids were also present they may have obscured any stimulatory action of GIP at a concentration of 1 ng/ml (Table IV).

Binding of GIP to a minority of the islet cell population was demonstrated in cultures stained with the immunoperoxidase method (13) following incubation of the culture with GIP (Fig. 1). Many of the islet cells and all of the fibroblasts remained unstained.

Discussion. Since both IRI and IRG secretion by these islet cultures were increased by physiologic amounts of GIP, a direct *in vitro* stimulatory effect of GIP on the pancreatic islet has been demonstrated. Previously, physiologic concentrations of GIP were shown to stimulate IRI secretion by the perfused rat pancreas, but very high and excessive concentrations were needed to increase IRI release by isolated islets (5, 6). This difference may be attributable to damage to GIP receptors when islets are isolated by the collagenase method. This is based upon the reported lack of inhibition of IRI secretion by somatostatin when examined in islets isolated by the collagenase method (14).

In preliminary reports, stimulation by

TABLE I. EFFECT OF AMINO ACIDS ON GIP-STIMULATED IRI RELEASE (μU/ml ± SD).[a]

Glucose (m*M*)	GIP (ng/ml)	With amino acids[b]	Without amino acids[c]
1.7	0	104 ± 8	79 ± 10
1.7	1	106 ± 13	89 ± 10
1.7	10	148 ± 19**	117 ± 22*
1.7	100	214 ± 43**	181 ± 16***
1.7	1000	283 ± 36***	–
16.5	0	141 ± 13	130 ± 29
16.5	1	173 ± 21*	220 ± 29**
16.5	10	309 ± 47***	381 ± 38***
16.5	100	536 ± 76***	757 ± 93***
16.5	1000	741 ± 65***	–

[a] *P* (compared to GIP, 0 ng/ml): (*) <0.05, (**) <0.01, (***) <0.001. *N* = 4 in all groups.
[b] EBSS supplemented with Eagle's amino acids (10).
[c] EBSS only.

TABLE II. EFFECT OF AMINO ACID SUPPLEMENTATION ON GLUCOSE-STIMULATED IRI RELEASE.

Glucose (m*M*)	IRI release with amino acids[a]/IRI release without amino acids[b]	*P*[c]
1.7	1.15	NS
5.5	1.11	NS
8.3	1.33	<0.01
16.5	1.50	<0.01

[a] EBSS supplemented with Eagle's amino acids (10).
[b] EBSS only.
[c] With amino acids vs without amino acids.

GIP of IRG secretion has been found both *in vivo* in man and *in vitro* with perfused rat pancreas (15, 16). Our findings also support these observations. Furthermore, this effect was not suppressible by high glucose concentrations. Both IRI and IRG release are modulated by the presence of an amino acid mixture, which also affects the relative stimulatory effect of GIP on these cultures. Interaction between glucose and amino acids has been previously shown for both IRI and IRG secretion by the perfused pancreas (17). Furthermore, we have indirect evidence from the studies reported here that GIP may interact with a minority subpopulation of islet cells (Fig. 1).

In pancreatic monolayer cultures, the secretion and/or localization of several hormones has been reported. These include, in addition to insulin (B cell) and glucagon (A cell), gastrin (D cell?) (18), somatostatin

TABLE III. EFFECT OF GIP ON IRG RELEASE (pg/ml±SD).[a]

ıg/ml)	Experiment 1[b] 1.7 m*M* Glucose	Experiment 1[b] 16.5 m*M* Glucose	Experiment 2[c] 1.7 m*M* Glucose	Experiment 3[c] 16.5 m*M* Glucose
	547 ± 110	400 ± 0	230 ± 38	230 ± 12
	653 ± 162	547 ± 55*	420 ± 78*	305 ± 78*
0	862 ± 48*	852 ± 33**	568 ± 125**	455 ± 30**

ɔmpared to GIP, 0 ng/ml): (*) <0.01, (**) <0.001.
3 in all groups.
4 in all groups.

.E IV. EFFECT OF AMINO ACIDS ON GIP-TED IRG RELEASE (pg/ml±SD) AT 8.3 m*M* GLUCOSE.[a]

N	No amino acids[b]	*N*	With amino acids[c]
3	110 ± 15	4	173 ± 13
4	146 ± 16*	4	193 ± 13
4	207 ± 15**	4	340 ± 36**

ɔmpared to GIP, 0 ng/ml): (*) <0.05, (**)

S only.
S supplemented with Eagle's amino acids

) (10), secretin (cell type?) (20), and ıtic polypeptide (cell type?) (21). ıtained by specific immunocytochem-thods, the majority of the islet cells e cultures appears to be B cells. er, when localization of exogenous ıs assessed by the methods used in 'estigation, only a small minority of :t cells was stained, suggesting that ells are either A or D cells, although ; by a subgroup of B cells is not :d. If the former is correct, then the of GIP on IRI secretion may not 'ia a direct action upon the B cells. findings suggest, therefore, that GIP t first on a cell type other than the B ıd its action on IRI release is, in ediated through this effect. This may ıugh direct cell-to-cell gap junctions r may be humorally mediated.

immunoperoxidase method used in dies reported here is less sensitive he peroxidase-antiperoxidase tech-ɔf Sternberger *et al.* (23). Using the ıethod and its much greater sensitiv-have recently observed the presence ' in the A cells of the unperfused al and adult rat pancreas as well as cells of 4-day-old monolayer cultures of pancreas from 2- to 5-day-old neonatal rats (24). Although GIP has been recently reported to inhibit the action of glucagon in rat adipocytes by competing with binding of glucagon to rat adipocyte receptors (25), we have observed in the studies using the peroxidase-antiperoxidase method that when GIP antiserum was incubated with glucagon prior to use, staining was still seen. Although the immunocytochemical findings reported here and our more recent observations with the peroxidase-antiperoxidase method may appear contradictory, there is a plausible explanation. Since the relative sensitivities of the two methods are so different, when islets are maintained in tissue culture for several days, much of the GIP within the A cells may become depleted to levels undetectable by the less sensitive procedure, but upon addition of exogenous GIP, that which is bound can be shown. However, with the peroxidase-antiperoxidase technique, we can demonstrate even low amounts of GIP. Thus, a tentative explanation for these findings is that exogenous GIP may not only bind to A cells but may also be taken up by these cells, presumably by endocytosis (26). An alternative explanation is that A cells may actually synthesize GIP.

Summary. Physiologic amounts of GIP stimulated the secretion of IRI and IRG by monolayer cultures of neonatal rat pancreatic islets. Localization of exogenous GIP to a minority subpopulation of the islet cells was observed. The results may be interpreted to indicate that the effect of GIP on IRI release by the B cell is not direct, but rather, is mediated through its action on another islet cell type. Conversely, the action of GIP on IRG release may be directly mediated through an effect of GIP on the A cell. The action of GIP on

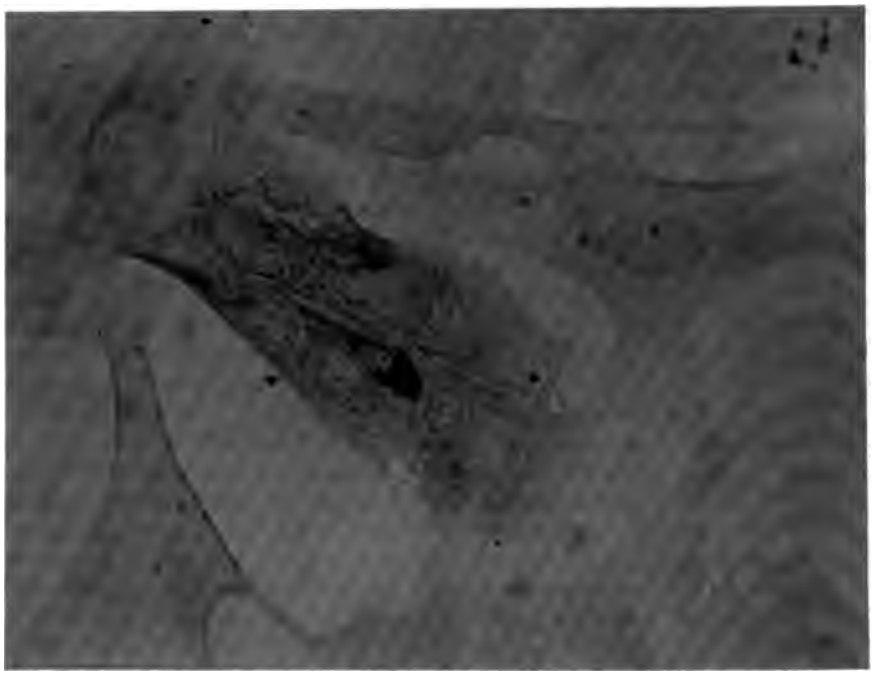

FIG. 1. Photomicrograph showing staining with the immunoperoxidase technique (13) following incubation of the culture with GIP. Strong staining is seen over one cell within the islet group, and lighter staining over two additional cells. The surrounding fibroblasts as well as the remaining islet cells are unstained. × 400.

IRG release apparently overrides any suppressive effect that high glucose levels may exert. The stimulation by GIP of both IRI and IRG release was greater when a mixture of amino acids was omitted.

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Effect of Freezing of Human Fecal Specimens upon the Isolation of Nuclear Dehydrogenating Clostridia (39998)

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There has been much interest in the possible role of intestinal bacteria in the etiology of colon cancer (1, 2). Coombs *et al.* (3) suggested that full aromatization of the bile acid nucleus would potentially yield a carcinogen metabolite based on cyclopentaphenanthrene. Goddard *et al.* (4) demonstrated the presence in the human fecal flora of nuclear dehydrogenating clostridia (NDC) which are capable of partially aromatizing a sterol nucleus.

Hill *et al.* (5) reported that a higher percentage of colon cancer patients had fecal NDC than did control patients. However, Finegold *et al.* (6) found no difference in counts of *Clostridium paraputrificum*, an organism capable of nuclear dehydrogenation, between patients with polyps and controls. Similar results were obtained by Moore and Holdeman (7) who showed that clostridial species are more numerous in the fecal floras of Africans, a low-risk population, than in the patients with polyps. Studies by Mastromarino *et al.* (8) indicate that species of *Clostridium*, although not the *C. paraputrificum* group, constituted a relatively greater percentage of fecal flora in patients with colon cancer.

The methods used by Hill *et al.* (5) for the isolation of NDC were different from those used by Mastromarino *et al.* (8), Finegold *et al.* (6), and Moore and Holdeman (7). The experimental techniques of Hill *et al.* (5) involved freezing the fecal specimens in glycerol soon after collection and performing NDC analysis at a later time, whereas methods used by Finegold *et al.* (6), Moore and Holdeman (7), and Mastromarino *et al.* (8) required fresh samples.

Freezing may be selective for NDC and thus accounts for the ability of Hill *et al.* (5) to show a correlation between colon cancer and NDC. We have, therefore, investigated the effect of freezing of fecal samples upon isolation of NDC.

Methods and Materials. Fecal samples were obtained in the New York Metropolitan area from healthy control persons and from patients with colon cancer and other colonic diseases. All of the subjects were eating a typical mixed Western diet. The feces were placed in a Gas Pack container (BBL) immediately after voiding and arrived under an anaerobic atmosphere at the laboratory for processing within 3 hr of the time that they were passed. Feces were processed under oxygen-free conditions in the laboratory. After thorough mixing of the fecal sample to obtain a homogenous sample, approximately 1.0 g wet wt of the fecal homogenate was suspended in 9.0 ml of glycerol broth (9). One milliliter of the suspension was removed, serially diluted, and plated on egg yolk lactose sugar (10). All dilutions were done under a gas mixture of 10% CO_2:5% H_2:85% N_2, and egg yolk plates were incubated under a 30% CO_2: 70% H_2 atmosphere (10). The remainder of the glycerol broth suspension was frozen and stored at $-20°$ for 2-3 weeks before processing. After thawing at room temperature, the frozen sample was processed in the same manner as the fresh samples. Several colonies, one colony of each morphological type or five colonies per plate, whichever yielded the highest number of colonies, were selected for determination of nuclear dehydrogenation ability. Colonies, after restreaking on egg yolk agar, were inoculated into Todd Hewitt broth and incubated, using the modification of Goddard *et al.* (4), with 5β-androsten-3,17-dione (Sigma) as the substrate and menadione (Sigma) as the electron acceptor. The cultures were extracted with chloroform and analyzed by thin-layer chromatography on silica gel GF plates, using solvent system A (benzene-dioxane-acetic acid 100:10:1), as described by Aries *et al.* (11). Fluorescence-

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ng spots corresponding in location rosten-3,17-dione were eluted from es and the uv absorption spectrum ermined, using a Beckman DG-GT hotometer. Only if the material had ption maximum of 240 nm, charac- of androstene, was the organism s being capable of nuclear dehydro- n.

s. Freezing the fecal samples in the broth medium affected both the d number of organisms obtained e suspension. Lecithinase activity in resh samples was very pronounced egg yolk agar. Because of overlap- nes of lecithinase activity, lecithin- tive and lecithinase-negative colo- ld not be differentiated. After freez- number of lecithinase-positive or- was greatly reduced, with most having no lecithinase-producing present. If lecithinase-producing were present, they were a minor ent of only 1 to 5% of the total on the plate. The number of col- ming units was also reduced by by a factor of 20- to 50-fold. In here there was a change in the of different types of colonies, as pon morphology, freezing resulted crease in morphological types rather a decrease.

e was also a greater frequency of n of nuclear dehydrogenating bacte- n frozen as compared to fresh stool , as shown in Table I. This table he number and percentage of per- om whom NDC have been isolated e number of persons in whom no ere detected. In comparing results esh samples and the same samples eezing and thawing, there is a signif- crease in the number and percentage ons from whom NDC were isolated I). The mean concentration of NDC from fresh feces is 2.4×10^7 bacte- et wt and 1.3×10^5 bacteria/g wet n frozen samples.

nies most likely to possess nuclear ogenation activity were flat irregu- ound colonies the color of which matched that of egg yolk agar me- nd a round concave colony with a bright pink color. Not all colonies matching these descriptions proved to be capable of dehydrogenating the androstan substrate used for assay purposes.

TABLE I. COMPARISON OF NUCLEAR DEHYDROGENATING CLOSTRIDIA IN FRESH AND FROZEN STOOL SAMPLES[a]

	NDC$^+$	NDC$^-$	NDC (%)
Fresh[b,*]	2	11	15
Frozen[c,*]	11	2	85

[a] NDC$^+$: Number of persons from whom NDC were isolated; NDC$^-$: number of persons from whom no NDC were isolated. NDC (%): percentage of group who harbor NDC.
[b] Sample assayed fresh upon arrival in laboratory.
[c] Sample assayed after freezing in glycerol broth.
* Significantly different by χ^2 test, $P < 0.0005$.

Discussion. Several changes occurred as a result of freezing fecal samples in glycerol broth. There was a 20- to 50-fold decrease in the number of colony-forming units, lecithinase activity was virtually eliminated, and there was an increase in the frequency of isolation of NDC from the primary egg yolk agar plates. The lecithinase-producing bacteria appear to be very sensitive to freezing. Although the NDC were not completely resistant to freezing, they were more resistant to freezing than were many of the other bacteria present in the fresh samples. Thus, freezing appears to be selective for NDC, resulting in a higher frequency of isolation of NDC after freezing.

Too little data have been collected at this time to form any conclusions about the effect of freezing upon correlation of NDC and colon cancer. We have found that freezing affects the frequency of isolation of NDC and are continuing to collect data to determine how freezing and NDC correlate with incidence of colon cancer.

Summary. Fresh and frozen human fecal specimens were used for the isolation of nuclear dehydrogenating clostridia (NDC). The total bacterial count and the number of lecithinase-positive bacteria were lower in frozen samples than in fresh samples, but the frequency of isolation of NDC from frozen specimens was higher than from fresh specimens.

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Variables Influencing Urinary pCO_2 during $NaHCO_3$ and Water Loading in Normal Man (39999)

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ion. During bicarbonate diuresis ubjects, urinary carbon dioxide O_2) is considerably greater than blood because in the distal neph- e of dehydration of the intralu- nic acid generated as a result of gen ion secretion is insufficiently mit CO_2 equilibration (1-9). s recognized that, in addition to n secretion, several factors in- ie pH, flow rate, and bicarbon- osphate levels influence urinary -6, 9-18), there is uncertainty their relative importance and ships. The present paper re- sults of multiple regression anal- elationship between urinary pH, '), bicarbonate and phosphorus n (U_{HCO_3} and U_P, respectively) n rates ($U_{HCO_3}V$ and U_PV, re- and urinary pCO_2 in normal ttempt to clarify the relative in- ese variables on urinary pCO_2.

and methods. Thirteen studies rmed in 12 healthy out-patient ages 21-51 years. Nine were three were female. None had aboratory evidence of renal, en- cardiovascular disease and all ing a regular diet.

lay of the study, 5% $NaHCO_3$ istered orally in a dose of 2 of body weight. This was given 5 min with an amount of water make the total fluid intake 500 was then collected under oil by s voiding at 20- to 30-min inter- urine pH exceeded 7.4, two or er urine collections were made ermination of the maximal urine lood pCO_2 difference. This was ure a consistently highly alkaline urine, thus avoiding misleading elevations of urine pCO_2 due to mixing of acid and alkaline urine in the urinary passages.

Immediately after measurement of the maximal difference between blood and urine CO_2 tension, seven of the subjects rapidly ingested 20 ml/kg body weight of water to obtain data extending over a wide range of urinary flow rates, pH, and urinary bicarbonate and phosphorus levels. Urine was collected serially every 20 to 30 min (approximately six observations per subject) for 2-3 hr. Arterial blood was not obtained after water-loading.

Written informed consent was obtained from each subject after explanation of the details of the procedures and any potential risks. The research was carried out according to the principles outlined in the Declaration of Helsinki and was approved by the Human Experimentation Committees of the University of Miami and the Miami Veterans Administration Hospital. No adverse effects occurred.

Determination of the pH and pCO_2 of blood and urine was completed within 5 min of collection on a radiometer Acid-Base Cart ABC-1. All urine and venous blood samples were measured for concentrations of sodium, potassium, phosphorus, and creatinine. In addition, the presence of glucose and protein was evaluated by reagent-impregnated plastic strips; neither was present in any of the samples tested. The analytical methods and calculations employed in our laboratory have been described previously (19, 20).

Statistical analysis. Statistical evaluation of the data was performed using both standard bivariate Pearson correlations and multiple regression analysis with a Univac 1106 at the computer center of the Univer-

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sity of Miami. The multiple regression linear model ($y = a_1x_1 + a_2x_2 + a_3x_3 + \cdots + B$) used was selected from a statistical package for the social sciences (SPSS) (21). Using the hierarchical method, R^2 change gives the component of variation attributable to the particular variable added in that step; the F ratio rates the independent influence of each variable when all variables have been introduced into the equation.

Results. The mean values of the variables influencing urinary pCO_2 ($UpCO_2$) following oral $NaHCO_3$ administration and prior to water-loading are given in Table I. The maximal difference between urinary and arterial blood pCO_2 was 47 ± 2 (SE) mm Hg, and arterial pCO_2 averaged 41 ± 2 mm Hg.

When the data of the pre- and post-water-loading periods were pooled (51 observations, 13 subjects), bivariate Pearson correlation analysis (Table II) showed a positive correlation between $UpCO_2$ an U_{HCO_3}, $U_{HCO_3}V$, and U_P, and a correlation with V ($P < 0.001$ There was no correlation between and U_PV or creatinine clearance. loading following bicarbonate-loa lowed analysis of the variables ove range of urine flow rate, pH, and b ate and phosphate levels. Figure the scattergrams illustrating these ships.

The individual regression lines of on V, UpH, U_{HCO_3}, and U_P for th subjects that underwent water-loa shown in Fig. 2. The overall tren individual patients was in the dire the changes indicated in Table II 1. Analysis of covariance showed cally significant differences betwee dividual slopes only in the case of on U_P and U_{HCO_3}.

Multiple regression analysis (Ta indicated that U_{HCO_3} (F ratio 11. 0.005) and U_P (F ratio 7.1, $P <$ were the only variables having a sig independent influence on $UpCO_2$. analysis, baseline and post-water observations were pooled. The F r the overall equation was 30.1 ($P <$

TABLE I. RESPONSE TO $NaHCO_3$-LOADING.[a]

	Mean ± SE	Range
$UpCO_2$ (mm Hg)	88 ± 2	78-102
Arterial pCO_2 (mm Hg)	41 ± 2	35-46
U-ApCO_2 (max) (mm Hg)	47 ± 2	32-59
Urine pH	7.84 ± 0.03	7.63-7.98
Urine Flow (ml/min)	1.7 ± 0.2	0.8-2.6
U_{HCO_3} (μequiv/ml)	168 ± 12	76-249
$U_{HCO_3}V$ (μequiv/min)	274 ± 35	82-457
U_P (μg/ml)	367 ± 87	36-1179
U_PV (μg/min)	566 ± 132	55-1650
C_{Cr}[b] (ml/min)	140 ± 12	100-216

[a] Values prior to water-loading at the time of the maximal urine to arterial blood pCO_2 difference (N = 13).

[b] Determined from the average of three to five collection periods.

TABLE II. BIVARIATE PEARSON CORRELATION ANALYSIS BETWEEN $UpCO_2$ AND VARIABLES ASSUMED TO INFLUENCE $UpCO_2$.[a]

	r	P value
$UpCO_2$ vs UpH	0.77	<0.001
$UpCO_2$ vs U_{HCO_3}	0.88	<0.001
$UpCO_2$ vs $U_{HCO_3}V$	0.45	<0.001
$UpCO_2$ vs U_P	0.53	<0.001
$UpCO_2$ vs V	−0.69	<0.001
$UpCO_2$ vs U_PV	−0.15	>0.2
$UpCO_2$ vs C_{Cr}	−0.12	>0.2

[a] Pooled data of pre (at the time of the maximal urine to arterial blood pCO_2 difference)- and post-water-loading periods.

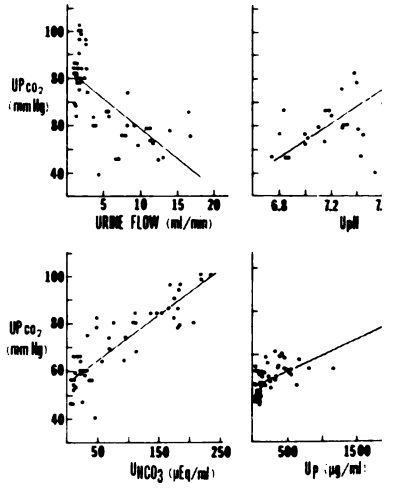

FIG. 1. Relationship between urinary ca ide tension ($UpCO_2$) and urinary pH (U flow rate, and urine bicarbonate (U_{HCO_3}) phorus (U_P) concentrations. The data of periods following water-loading were poole baseline values obtained at the time of det of maximal U-ApCO_2 (51 observations, 13

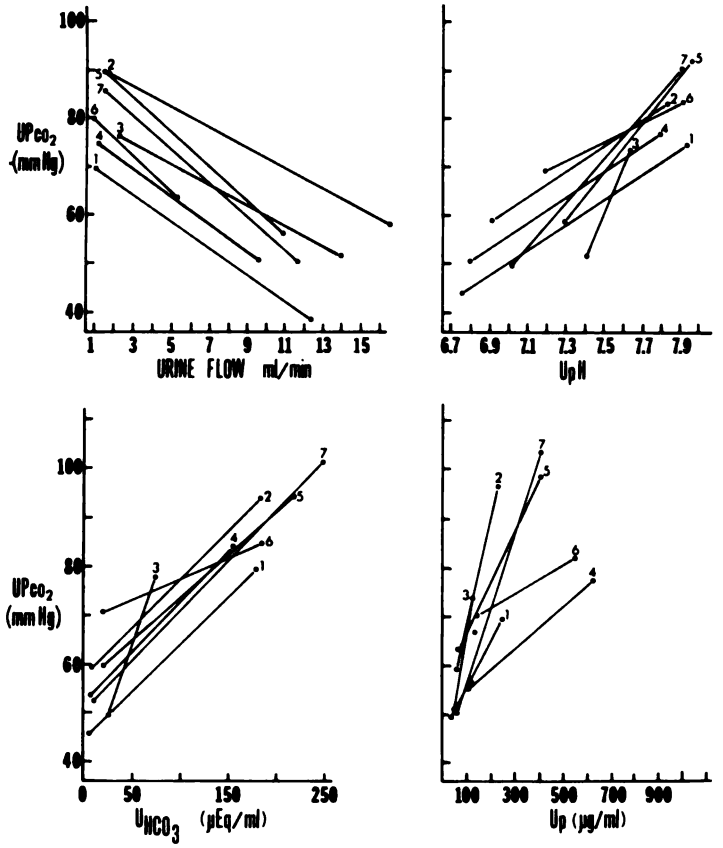

FIG. 2. Individual regression lines of $UpCO_2$ on V, UpH, U_{HCO_3}, and U_P for the seven subjects that underwent water-loading. Analysis of covariance showed statistically significant differences between the individual slopes only in the case of $UpCO_2$ on U_P and U_{HCO_3}.

TABLE III. MULTIPLE REGRESSION ANALYSIS OF FACTORS AFFECTING URINARY pCO_2.[a]

	U_{HCO_3} μequiv/ml	U_P μg/ml	$U_{HCO_3}V$ μequiv/min	C_{Cr} ml/min	UpH	V ml/min	U_PV μg/min
Range	4–249	36–1179	40–455	69–232	6.66–7.98	0.8–17	55–1650
R^2 change[b]	0.77	0.03	0.02	0	0	0	0
F ratio[c]	11.6	7.1	4.0	3.2	0.4	0.3	0
P[c]	<0.005	<0.025	NS[d]	NS	NS	NS	NS

[a] Pre- and post-water-loading data pooled (only 46 observations were included because C_{Cr} was not available in five H_2O loading periods in one patient).

[b] R^2 change indicates the component of variation attributable to the particular variable added in that step (hierarchical method).

[c] F ratio and corresponding P value rate the variables as to their independent influence when all variables have been introduced into the equation.

[d] Not statistically significant ($P \geq 0.05$).

The regression equation ($UpCO_2 = 0.13\ U_{HCO_3} + 0.02\ U_P - 0.06\ C_{Cr} + 0.04\ U_{HCO_3}V - 4.9\ UpH - 0.3\ V + 96$) derived from the data obtained in our patients predicts the values of urinary pCO_2.

Discussion. There are very few detailed data available in the literature concerning the maximal urine to blood pCO_2 difference in normal subjects (20, 22). In the present study, the response to oral $NaHCO_3$-loading was evaluated in 13 studies in 12 healthy subjects, and the findings confirm and am-

plify those of Halperin *et al.* (22) and are in agreement with our earlier observations (20).

Seven of the subjects underwent oral water-loading immediately following determination of maximal U-ApCO_2. Using standard bivariate analysis a positive correlation was noted between UpCO_2 and U_{HCO_3}, $U_{HCO_3}V$, UpH, and U_P, and an inverse correlation betwen UpCO_2 and V, over a wide range of urine flow rates, pH, and bicarbonate and phosphorus levels (Table II). Multiple regression analysis suggested that urinary bicarbonate and phosphorus concentrations were the predominant influences on UpCO_2 and that the independent influences of the other variables including U_PV, $H_{HCO_3}V$, and V were small. It is important to point out, however, that multiple regression analysis as utilized in this study, like other similar statistical techniques, can only indicate significant correlations between variables and cannot prove a cause and effect relationship.

Since the recent suggestion that U-ApCO_2 can be used as a sensitive qualitative index of distal nephron hydrogen ion secretion (22), there has been an upsurge of interest both in man (20, 23-25) and experimental animals (15-18, 26-28) in the determination of urinary pCO_2 during alkali-loading.

According to Halperin *et al.* (22), when the intraluminal pH is greater than that of the arterial blood neither back-diffusion nor gradient limitation of hydrogen ion secretion is operative when the urinary pCO_2 fails to increase appropriately after alkali administration. These investigators, therefore, have interpreted the finding of low U-ApCO_2 in patients with distal renal tubular acidosis (RTA) as indicative of decreased formation of H_2CO_3 (and defective cellular hydrogen ion secretion). In contrast, the supranormal bicarbonate T_m noted in some patients with classical distal RTA (29) and the finding that these patients tend to excrete less bicarbonate than alkali-loaded controls suggested to Sebastian *et al.* (23) that H_2CO_3 generation is not abnormal in this setting. Rather, these investigators suggested that rapid back-diffusion of H_2CO_3 accounts both for the low U-ApCO_2 and for obliteration of the dise pH which facilitates bicarbonate tion (23). Sebastian *et al.* (23 therefore, that the distal luminal r allows hydrogen ion back-diffus the pH of the luminal contents i H_2CO_3 back-diffusion when the p This explanation, however, has l cized by Stinebaugh *et al.* since th demonstrated that the presumed of the distal tubular disequilibriu carbonic anhydrase infusion did nc tubular reabsorption of bicarbona

The urinary pCO_2 is influenced variables in addition to distal tubu gen ion secretion (2, 4-6, 9-18). I that elevated urine pH and bicarb phosphate levels, and low urine to increase urinary pCO_2. Thus, forming bicarbonate-loading studi detection of defects of distal tubul cation it is desirable that these va comparable in the control and exp groups. Bicarbonate serves as the for H_2CO_3 generation (1-3), and gen phosphate ($H_2PO_4^-$) delays dration of H_2CO_3 (13, 31) and hydrogen ion which back-titrates bonate in low-flow, bicarbonate- (6).

Several investigators have show maximal elevation of UpCO_2 is with high urinary bicarbonate le 10, 11) and that urinary pCO_2 te elevated when phosphate levels either spontaneously (11) or follo sions of phosphate (4, 5, 11). A tends to increase the UpCO_2 o urine (6, 10, 11), perhaps by incr concentration of buffer and bic thus, facilitating the back-titrati latter as the pH rises (6). The f the present study using bivariat (Table II) are in good agreement concepts, since they show that flow rate and high urinary levels phate and/or bicarbonate are with higher urinary CO_2 tension. Henderson-Hasselbalch equation that under steady-state conditions urine pH is relatively fixed, the p be directly related to the bicarbo centration, it was expected that the

ɔrrelation between urinary pCO_2 and ɔnate concentration in the present Multiple regression analysis was help- ıwever, in assessing the influence on / pCO_2 of the various other variables very wide range of values produced nbined bicarbonate and water-load-

ɔite the above-mentioned studies, the ɛ influence of urine flow and urinary ıate and bicarbonate levels on urinary is unclear. During bicarbonate-load- ı elevated urinary pCO_2 obtains with nt levels of bicarbonate excretion (4, ıd Thirakomen *et al.* (33) have stated ry low bicarbonate excretion in bicar- -loaded experimental animals with inferior vena cava constriction does ɔear to limit the rise in urinary pCO_2. ough Kennedy *et al.* (11) demon- a direct correlation between pCO_2 ffer concentration in the urine, other are in conflict with this notion. Port- ɛt *al.* (4) demonstrated that urinary during $NaHCO_3$ diuresis can greatly the pCO_2 of the arterial blood even urinary phosphorus (buffer) is very ıd Dorman *et al.* (2) showed that a ɨinary pCO_2 can occur in the presence ɛry low phosphate buffer concentra- · excretion rate. Portwood *et al.* (4) ded that the excretion of buffer, al- influencing urine CO_2 tension to ɨxtent, has only a minor effect in the ɔf buffer excretion ordinarily encoun- Moreover, Thirakomen *et al.* (33) y demonstrated in bicarbonate-loaded ith maximally alkaline urine that the ion of urinary pCO_2 was uninflu- by urinary phosphate concentrations. authors suggested that when urine is ally alkaline almost all phosphate is HPO_4^{2-} form and that, as a conse- ɨ, its buffer effect (and thus its influ- ı urinary pCO_2) is minimal.

ough several investigators have an inverse relation between urine ınd flow rate (6, 10, 11), Rector *et* and Portwood *et al.* (4) also demon- that $UpCO_2$ can remain elevated high urine flow rates as long as (and presumably distal delivery of remains high.

The findings in the present paper are in agreement with other studies suggesting that urinary bicarbonate, and to a lesser extent, urinary phosphate concentrations are the important determinants of the CO_2 tension of alkaline urine. In the range of values noted, V, $U_{HCO_3}V$, U_PV, and creatinine clearance appeared to have no important independent influence on urinary pCO_2.

Summary. In the present study the relative influence of several variables, other than distal renal tubular hydrogen ion secretion, on urinary pCO_2 was evaluated. Thirteen studies were conducted in 12 healthy volunteers aged 21–51 years who received 2 mequiv/kg body weight of $NaHCO_3$ orally. When urine pH exceeded 7.4, urinary pCO_2 and arterial blood pCO_2 were measured. The maximal difference between urine and arterial blood pCO_2 was 47 ± 2 (SE) mm Hg. In order to obtain data extending over a wide range of urine flow rates, pH, and bicarbonate and phosphorus levels, seven subjects subsequently ingested 20 ml/kg body weight of water. Bivariate Pearson analysis (pre- and post-water-loading data pooled) showed a positive correlation between $UpCO_2$ and the following variables: UpH ($r = 0.77$), U_{HCO_3} ($r = 0.88$), $U_{HCO_3}V$ ($r = 0.45$), and U_P ($r = 0.53$) ($P < 0.001$ for all). A negative correlation was obtained between $UpCO_2$ and V ($r = -0.69$, $P < 0.001$). Multiple regression analysis suggested that U_{HCO_3} and U_P were the predominant influences on $UpCO_2$ and that the independent influences of the other variables were small and not statistically significant.

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ro Effects of Melatonin and Serotonin on Adrenal Steroidogenesis[1] (40000)

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ioval of the ovaries in rats results in ed adrenal secretion of the principal olites of corticosterone (5α-dihydro-sterone, DHB; and 3β,5α-tetrahy-ticosterone, THB) with a proportion-in the production of corticosterone 'e recently reported that the pineal modifies the elevated rate of DHB HB formation observed after ovariec-(2). Secretion of these metabolites s after pinealectomy and proportion-tput of corticosterone is restored *in* id *in vitro*. Administration of mela-*n vivo* reverses the effects of pineal-by stimulating the secretory rate of and THB with a concomitant decline portionate output of corticosterone drenal effluent (3). These changes ediated by changes in adrenal 5α-ase activity. Pinealectomy suppresses ase activity whereas melatonin stimu-ctivity of the enzyme. The indole is effective in hypophysectomized and ctomized rats (3). Thus, melatonin ntly does not require participation of terior pituitary gland or the pineal or its effects on adrenocortical func-has often been presumed (4). The t investigation defines more clearly ect action of melatonin by incubating l adrenal tissue with the indole.

rials and methods. Female Long-Ev-s were maintained under a constant eriod of 12-hr light: 12-hr dark. A ature of 22 ± 0.5° was maintained. y 42 of age rats were rapidly decapi-etween 0830 and 0930 hr, adrenals moved and placed in ice-cold Krebs-bicarbonate solution (KRB). Adre-nal 5α-reductase activity was assayed in whole adrenal homogenates. Adrenal tissue was incubated in air at 37° with 25 μg of corticosterone in a final volume of 0.25 ml for 60 min. Details of reaction mixture and other procedures have been presented (4). Rate of disappearance of added steroid was determined by the loss of fluorescence, shown previously to coincide with the formation of 5α-reduced metabolites (1, 6). Adrenal slices were incubated at 37° in 2 ml of KRB under an atmosphere of 95% O_2-5% CO_2. After a 30-min preincubation period, the medium was discarded. Fresh KRB was added for a final incubation of 60 min.

Melatonin and serotonin was added to incubation media to achieve a final concentration of 45 nmole/ml. A corresponding volume of saline vehicle was added to the control incubates. After incubation, media of both homogenates and slices were extracted with chloroform, and corticosterone was measured by acid fluorescence (7). Total corticosteroid production was evaluated by the blue tetrazolium reaction which is positive for steroids and an α-ketol side chain (8). In one experiment corticosterone and its principal metabolites, 5α-dihydrocorticosterone (DHB) and 3β,5α-tetrahydrocorticosterone (THB), were measured specifically by competitive protein binding assay (9) subsequent to isolation by paper chromatography (10). Identification and homogeneity of these compounds have been established by paper chromatography, thin-layer chromatography, infrared spectroscopy (1), and mass spectrometry (10).

Procedures of statistical analysis were selected according to comparisons planned for each experimental design (11). Bartlett's test was used to evaluate homogeneity of variance and log transformations were made when indicated. Multiple comparisons with control values were made by the Dunnett

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test, and single comparisons, by Student's *t* test. The Behrens–Fisher modification of the latter was used when heterogeneity of variance was encountered.

Results. Adrenal 5α-reductase activity (Table I) is stimulated by incubating adrenal homogenates in the presence of melatonin. Total steroid secretion by adrenal slices is also enhanced without a specific change in corticosterone secretion. Consequently proportionate secretion of corticosterone declined.

The next experiments were designed to ascertain whether serotonin mimicked the *in vitro* effects of melatonin. Table II indicates that serotonin has no effect on adrenal 5α-reductase activity but the stimulatory effect of melatonin is reproduced. Melatonin specifically enhances adrenal slice secretion of the principal reduced metabolites of corticosterone (DHB and THB) with a concomitant decline in corticosterone output (Table III). Proportionate secretion of corticosterone is likewise reduced. Serotonin is entirely ineffectual in altering these parameters.

Discussion. In 1970 Giordano *et al.* (12) found that the melatonin impaired adrenal homogenate production of Δ4-3-ketosteroids. Although the mechanism mediating this defect was unclear, they determined that it was not due to alterations in Δ5,3β-hydroxysteroid dehydrogenase activity. Our findings extend these observations to provide the mode of action. Melatonin enhances secretion of A ring-reduced metabolites by direct stimulation of 5α-reductase activity. This can account for the observed decline in Δ4-3-ketosteroids without corresponding alterations in Δ5,3β-hydroxysteroid dehydrogenase activity. However, in our hands the effect of melatonin on corticosterone production per se is variable; corticosterone output was not altered by melatonin in one experiment (Table I) but was greatly diminished in another (Table III). The basis for the variability is unclear. Nevertheless, presence of melatonin consistently resulted in decremental changes in proportionate output of corticosterone which are specifically associated with increased secretion of DHB and THB. The identical alterations in corticosteroidogenesis also occur when melatonin is administered *in vivo* to intact rats or subsequent to pinealectomy, ovariectomy, or hypophysectomy (3). Thus, melatonin exerts its effects directly on the adrenal cortex rather than being mediated by secondary influences. This implies that melatonin-specific receptors may exist in the adrenal. Kopin and co-workers (13) found the turnover rate of melatonin in the adrenal to be about one-half that of other organs which is consistent with existence of specific receptors. In addition 90–95% of the melatonin taken up by the adrenal is localized in the microsomal-cell sap fraction. Such conditions provide adequate opportunity for melatonin–reductase interaction since the enzyme is specifically localized in the microsomal fraction (14). The mechanism by which melatonin stimulates activity of this

TABLE II. *IN VITRO* EFFECTS OF MELATONIN AND SEROTONIN ON ADRENAL 5α-REDUCTASE ACTIVITY.[a]

	Reductase activity[b]
Saline (7)	0.06 ± 0.20
Melatonin (8)	2.01 ± 0.56*
Serotonin (8)	0.22 ±0.51

[a] Values are means ± SE. Number of animals indicated within parentheses.
[b] Micrograms of corticosterone reduced per 10 mg adrenal weight per hour.
* Mean differs from control value, $P < 0.05$.

TABLE I. *IN VITRO* EFFECTS OF MELATONIN ON ADRENAL STEROIDOGENESIS[a]

	Reductase activity[b]	Corticosterone secretion[c]	Total steroid secretion[c]	Corticosterone/total steroids (%)
Saline (10)	0.55 ± 0.28	3.1 ± 0.6	6.9 ± 0.9	55.3 ± 3.3
Melatonin (10)	2.12 ± 0.40***	4.6 ± 0.5	12.4 ± 2.6*	35.3 ± 8.3**

[a] Values are means ± SE. Number of animals indicated within parentheses.
[b] Micrograms of corticosterone reduced per 10 mg adrenal weight per hour.
[c] Micrograms per mg adrenal weight per hour.
* Means differ from control value, $P < 0.05$.
** Means differ from control value, $P < 0.01$.
*** Means differ from control value, $P < 0.001$.

ABLE III. *In Vitro* Effects of Indoleamines on Steroidogenesis in Adrenal Slices.[a]

nent	Corticosterone secretion[b]	Dihydrocorticosterone secretion[b]	Tetrahydrocorticosterone secretion[b]	Corticosterone/total steroids (%)
3)	3.90 ± 0.81	2.72 ± 0.37	0.35 ± 0.03	47.7 ± 3.1
in (8)	1.95 ± 0.26**	3.51 ± 0.29*	0.50 ± 0.07*	33.1 ± 2.7***
in (8)	3.41 ± 0.81	2.11 ± 0.31	0.43 ± 0.06	54.8 ± 3.2

are means ± SE. Number of animals indicated within parentheses.
grams per 100 mg adrenal weight per hour.
larger than control value, $P < 0.05$.
smaller than control value, $P < 0.025$.
n smaller than control value, $P < 0.005$.

is unknown. It probably involves nent of protein synthetic mecha- ce cycloheximide prevents the rise ase activity after hypophysectomy elatonin appears to be a potent r of adrenal 5α-reductase activity. latory effect stands in striking con- l all other hormones so far studied. prolactin, GH, testosterone, and inhibit 5α-reductase activity (1, 6, whereas melatonin is the only hor- own to exert a stimulatory influ-

ary. The effects of melatonin and l on adrenal steroidogenesis were Adrenals were obtained from 42- intact female Long-Evans rats ed under a photoperiod of 12-hr hr dark. Tissue slices and homoge- re incubated in the presence of 45 l of melatonin or serotonin. Mela- mulates reductase activity. As a nce secretion rates of dihydrocor- ne and tetrahydrocorticosterone in- th a concomitant decline in propor- secretion of corticosterone. Sero- s not alter any of these parameters al steroidogenesis. ACTH, prolac- , testosterone, and estradiol have n known to inhibit 5α-reductase The stimulatory influence of mela- nds in striking contrast to the ef- hese hormones.

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Antenatal Diagnosis of Cretin Lambs by Measurement of Amniotic Fluid Thyrotropin[1] (40001)

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Documentation of fetal hyper- or hypothyroidism before birth by measurement of amniotic fluid thyroxine (T_4) iodine by column or triiodothyronine (T_3) by radioimmunoassay (1, 2) has been difficult. Persistent, long-term postnatal effects of insufficient (3, 4) or excessive (5) levels of thyroxine *in utero* have been described in children. Since these developmental problems may not be fully reversed by treatment after birth, the search continues for feasible diagnostic measures to detect thyroid disorders *in utero*. These experiments were undertaken in sheep to investigate the possibility that amniotic fluid thyrotropin (AFTSH) and 3,3',5'-triiodothyronine (reverse T_3; rT_3) levels might reflect fetal hypothyroidism.

Materials and methods. Nine crossbred Hampshire, Suffolk, and Rambouillet ewes with dated matings were obtained from the University of Kentucky Ovine Research Farm and maintained at environmental temperatures of 22–24° with free access to water and Purina Omolene grain containing iodized salt. *In utero* fetal thyroidectomy was performed in six pregnant ewes at 50–93 days of gestation by the microsurgical technique previously described (2). Completeness of thyroidectomy was assessed in all experimental animals by later postmortem dissection and histological examination of serial sections of the neck. Eleven normal lambs were studied as controls. These included four twin pregnancies in which only one fetus was subjected to thyroidectomy, with the nonoperated twin in the contralateral noncommunicating uterine horn s as a control. In the three remaining nancies in which no fetal thyroidec were performed, there were two s normal twin lambs and one set of r triplets.

At the time of surgery amniotic flu aspirated preoperatively from each nc municating horn of all animals for TS and T_3 determinations. At Caesarea tion amniotic fluid was aspirated w 18-gauge needle by amniocentesis each uterine horn before rupture of branes and delivery. In two pregr with cretin lambs (one twin pair w unoperated control) and three norma nancies (seven animals including two twins and one set of triplets), rT_3 de nations were also performed.

Delivery was accomplished by Cae section at term (143–147 days in this under general anesthesia. Neonatal j blood was drawn after cord cutting 10–60 min of delivery for TSH, r and T_4 determinations. Timing of ne hormone measurements in twins was 5 min in control and experimental ar

T_4 was measured by the method o sler and Pileggi (6), whereas T_3 was mined by the method of Larsen (7) measurements were done by the met Borger and Davis (8). The concentra rT_3 was measured in ethanol extr amniotic fluid by a previously des radioimmunoassay (9, 10). Cortisol in amniotic fluid were determined method of Mattingly (11) in five r and two cretin lambs. *P* values for sta analysis of the data were obtained b dent's *t* test.

Results. Completely thyroidectc lambs showed the cretinoid habitus

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lescribed (2). When thyroidectomy s than total, partial cretins had milder :a of congenital hypothyroidism (2). e I summarizes the thyroid hormone yrotropin findings in amniotic fluid ed at term, and in neonatal lamb immediately after delivery.

iiotic fluid studies. Amniotic fluid ob- at surgery during the middle third of ncy did not contain measurable ts of TSH, rT_3, T_3, or T_4.

:rm, very low levels of T_4 were found mal animals (range, 0.8–2.8 μg/dl) :etins (range, 0.1–1.0 μg/dl). The nce was significant ($P < 0.05$), but) in the two groups prevented diag- accuracy.

as not detectable in our assay system n in any pregnancy. Reverse T_3 in regnancies in two cretin animals and)rmal controls was <10 ng/dl. In one pregnancy with three normal animals evel of 13 ng/dl of rT_3 was measured iotic fluid from one uterine horn and /dl from fluid in the contralateral : horn containing two fetuses.

'SH was present in low levels (mean, /ml) in 10 normal animals at term. In st, four cretin animals had a mean AFTSH value of 54 ng/dl.

At postmortem examination two additional cretin lambs (28B and 234A) had tiny thyroid remnants (ca. one-quarter normal size). In both instances AFTSH was higher in these partial cretins than in their normal twins (9.3 vs 2.9 ng/ml in 28B and 28A, 4.1 vs 0.7 ng/ml in 234A and 234B; see Table I).

Amniotic fluid cortisol measurements were obtained at term in five normal and two cretin pregnancies. The mean values of 1.6 μg/dl in normals and 1.3 μg/dl in cretins were similar.

Thyroid hormones and thyrotropin in neonatal lamb serum. Normal newborn lambs within 60 min of Caesarean section had a mean T_4 level of 5.4 μg/dl as compared with only 1.2 μg/dl in cretin animals. Aside from one partial cretin (234A) with a level of 4.1 μg/dl (small thyroid remnant was found at autopsy in this case), four athyrotic cretins and one partial cretin had T_4 measurements of <1.0 μg/dl at birth.

T_3 in normal lambs ranged from <25 to 293 ng/dl 10–60 min after birth, with a mean of 114 ng/dl. In contrast, in all cretin lambs values were <70 ng/dl, with a mean value of 37 ng/dl. There was overlap in the

\BLE I. Amniotic Fluid and Newborn Serum Studies of Thyroid Function in Lambs after Total or Incomplete Thyroidectomy *in Utero*.

	Amniotic Fluid				Serum			
	TSH (ng/ml)	T_4 (μg/dl)	rT_3 (ng/dl)	Cortisol (μg/dl)	TSH (ng/ml)	T_4 (μg/dl)	rT_3 (ng/dl)	T_3 (ng/dl)
	3.7 (10)[a]	1.6 (9)	<10 (7)	1.6 (6)	1.9 (11)	5.4 (11)	511 (8)	114 (11)
	0.73	0.32	2.5	0.2	0.29	0.51	43	28
yroidec- y								
	117	0.8	–	–	210	0.9	–	25
	13	0.7	<10	1.3	472	0.8	<10	27
	63	0.2	<10	1.3	443	0.4	<10	30
	21	0.6	–	–	592	0.6	–	45
:te thy- lectomy								
	9.3	1.0	–	–	136	0.7	–	29
	2.9	0.9	–	–	2.4	4.9	–	161
l twin								
	4.1	0.1	–	–	16	4.1	–	69
	0.7	0.1	–	–	2.1	4.9	–	58
l twin								
	<0.01[b]	<0.05	NS	NS	<0.005	<0.005	<0.005	<0.1

ıbers of determinations are in parentheses.

lues compare normal animals with those confirmed to have total thyroidectomy.

two groups, and the difference between them was not significant ($P < 0.1$).

Serum rT_3 levels were measured in two cretin lambs and found to be <10 ng/dl. Eight normal lambs had very high levels of serum rT_3 at birth, ranging from 400 to 740 ng/dl, with a mean of 511 ng/dl ($P < 0.005$). The high rT_3 levels in normal newborn lambs were in sharp contrast to the mean ewe rT_3 level of 50 ng/dl (range, 24–85 ng/dl) in simultaneously drawn samples from the maternal jugular vein.

TSH levels were low in normal lambs immediately following Caesarean section (mean, 1.9 ng/ml). This was in marked contrast to the mean value of 312 ng/ml in cretin lambs ($P < 0.005$).

All athyrotic lambs and one animal with incomplete thyroidectomy (28B) died of pulmonary failure on the day of birth in spite of intensive neonatal care. Lamb 234A (partial thyroidectomy) died on Day 1 during cardiac function studies.

Discussion. Previous studies in sheep have shown minimal maternal-to-fetal transfer of T_4 and T_3 (12–14). Chopra *et al.* have reported significant levels of rT_3 in human cord blood and in serum of normal newborn lambs (9, 15). It has not been previously possible to document congenital hypothyroidism antenatally in human infants or lambs by measurement of decreased T_3 or T_4 in amniotic fluid (1, 2). Measurements of T_4 in amniotic fluid have not been clearly diagnostic because of the low levels normally present, and T_3 is not detectable in human or ovine amniotic fluid with present assay methods.

In newborn lambs the higher serum T_3 values after cord cutting may reflect the alteration in T_4 to T_3 conversion and the TSH surge which occur within the first hour of life (16).

These studies were undertaken in our well-established model for cretin lambs with the hope that AFTSH and rT_3 might provide a useful diagnostic tool for antenatal thyroid diagnosis. Unexpectedly, rT_3 was not found in ovine amniotic fluid. Ovine amniotic fluid was clearly very different from human amniotic fluid with respect to rT_3 concentration. Very high levels of rT_3 were found, however, in the serum of normal newborns, as observed previously (15). Similarly, as expected, serum rT_3 levels were found to be very low (undetectable) in two newborn cretin lambs. One of these animals had a normal twin in the contralateral horn. This newborn lamb had a serum rT_3 level of 470 ng/dl at birth. The reasons for differences in rT_3 concentration in ovine amniotic fluid compared to human amniotic fluid are not clear. There is a significant difference, however, in placentation between sheep and man. The sheep placenta consists of a group of separate cotyledons instead of a single mass of placental tissue. The information so far available does not suggest a major difference in T_4 monodeiodination between man and sheep (15).

In contrast, significantly elevated levels of AFTSH clearly predicted the birth of athyrotic lambs. The findings in twins were particularly impressive, and in four instances AFTSH clearly distinguished the severely hypothyroid fetus. In two lambs with incomplete thyroidectomy, AFTSH levels were higher than those in their normal twins. When sufficient residual thyroid tissue was present to provide a normal neonatal serum T_4 level (lamb 234A), amniotic fluid values were less helpful diagnostically than neonatal serum TSH levels. Amniotic fluid TSH levels correlated with the severity of hypothyroidism.

Summary. Elevated TSH levels are highly reliable in predicting neonatal lamb hypothyroidism. However, the method is not as sensitive as serum TSH measurement, and mild hypothyroidism can exist with normal amniotic fluid thyrotropin and elevated serum TSH.

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Oxonate-Induced Hyperuricemia and Orotic Aciduria in Mice (40002)

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Hyperuricemia and uricosuria are the most striking characteristics of gout. Painful inflammation in the joints (tophi) caused by deposits of urate classify this disease as a form of arthritis. The formation of renal calculi and progressive renal failure are also common complications (1, 2). In most mammalian species the enzyme uricase catalyzes further oxidation of uric acid to the more soluble allantoin. However, in humans this enzyme is lacking and probably accounts for the difficulty in handling large purine loads.

The presence of uricase activity in experimental animals has complicated their use as models for studying gout. In 1965, Fridovich (3) examined various chemical compounds bearing a structural resemblance to the pyrimidine portion of the purine ring of uric acid and xanthine as possible inhibitors of *in vitro* uricase activity. These studies revealed that potassium oxonate (KOx; 2,4-dihydroxy-6-carboxy-1,3,5-triazine) was a potent inhibitor of *in vitro* hog liver uricase. *In vivo* experiments with rats fed a diet supplemented with 5% KOx were shown to produce a profound hyperuricemia and uricosuria (4). These changes in blood and urine urate were further increased when the diet was supplemented with an additional 1% uric acid. Hyperuricemic nephropathy was first demonstrated in rats fed 5% KOx plus 1% uric acid (5). Subsequent experiments revealed that a diet containing 2% KOx supplemented with 3% urate was sufficient to induce the hyperuricemic nephropathy (6, 7). Several studies using dogs, rabbits, pigs, and mice have reported the inhibitory effects of KOx on purine metabolism (8-11).

The present studies were conducted to more clearly define the effects of graded dietary concentrations of KOx with or without urate supplementation upon hyperuricemia, uricosuria, and orotic aciduria. Comparisons are made between these data and published data on the rat fed KOx.

Materials and methods. Forty ma bino ARS/ICR[1] mice (19-25 g) adapted for 3 days to a semipurified diet suspended in 2% agar gel. Diet sisted of 50% water and 50% dry mi dry mix was composed of 15% vitam casein, 0.3% methionine, 5% corn o mineral salt mix,[2] 1% vitamin mix,[3] cornstarch, and 24.6% sucrose.

All animals were randomly assigne the adaptation period to one of eight ment groups. The experimental diet sisted of KOx incorporated at con tions of 0, 1, 3, and 5%, with or w 1% supplemental uric acid. Oxona urate were added at the expense of proportion of cornstarch and sucrose and water were provided *ad libitum*. V changes were determined perio throughout the 15 days of experi feeding. Mice were identified by ear ing and housed five to a cage, exc purposes of urine collection. During collections, animals were housed i ually in stainless steel metabolism Food intakes were determined in in ally housed animals.

KOx was either purchased comme or synthesized accordingly to the met Brandenberger (12). Chemical puri established by thin-layer chromato using an *n*-butanol:acetic acid:water s system (5:1:2) and by chemical anal carbon and nitrogen. The commercia had the following composition: C,

[1] ARS/Sprague-Dawley, P. O. Box 4220, M Wisc. 53711.

[2] Rogers and Harper salt mixture No. Teklad Test Diets, 2826 Latham Drive, M Wisc. 53713.

[3] Vitamin fortification mix No. 40060, Tek Diets, 2826 Latham Drive, Madison, Wisc. 53

[4] Oxonate, potassium salt, A grade No. Calbiochem, 10933 N. Torrey Pines Road, I Calif. 92037.

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N, 21.60. KOx synthesized in our
ry was found to contain C, 24.38;
; and N, 21.31. Synthesized KOx
ed *in vitro* for inhibition of uricase
The synthesized oxonate was found
potent competitive inhibitor of uri-
h a K_I similar to that previously
(3).
ied urine was collected from three
y selected mice from each treat-
Days 7 and 14 of feeding. Urinary
mmonia, creatinine, and orotic acid
alyzed according to the methods of
nd Hochholzer (13), Chaney and
n (14), Folin and Wu (15), and
et al. (16), respectively. Urinary
l was determined by the method of
ld (17) and confirmed by high-pres-
uid chromatography (hplc).[5] Blood
l by heart puncture was also ana-
r urate by hplc.[5]
e time of sacrifice, spleens, livers,
neys were weighed and stored at
r subsequent analysis. Portions of
and liver were homogenized in 0.4
and centrifuged at 3730*g* for 10
sue uric acid content was analyzed
ricase activity of these tissues was
ned spectrophotometrically as de-
by Fridovich (3). Tissue protein was
ned colorimetrically using bovine se-
umin as a standard (18).
ical methods. All data were ana-
atistically by analysis of variance,
mparisons were made by using Dun-
ultirange test (19). A *P* value of
less was considered statistically sig-
.

ts. Growth data are presented in
. There were no significant altera-
growth of mice fed 1 or 3% KOx.
r, growth was severely inhibited by
x. Although animals consuming the
x diet grew slightly with time, their
was still severely inhibited at the
he feeding period. Uric acid supple-
on alone did not significantly influ-
owth. However, uric acid supple-
on to diets containing oxonate de-
growth more severely than diets
containing only oxonate. During the first week there was a 50% depression in growth with 1% dietary KOx plus 1% urate. Growth was further inhibited with 3 and 5% oxonate supplemented with uric acid. Similar growth data was obtained after 2 weeks of feeding.

Feed intake was significantly inhibited by supplementation of the diet with 5% KOx (Table I). Animals receiving supplemental uric acid showed further reductions in feed intake. Some adaptation to the diets appears to have occurred during the feeding period since intakes increased with time. However, feed consumption by mice consuming 3 and 5% KOx plus urate remained significantly depressed throughout. The differences in feed consumption are not of sufficient magnitude to account entirely for the severe depressions or inhibition of growth observed with supplementation of KOx. Increasing the dietary content of oxonate resulted in a marked reduction in feed efficiency. This reduction in feed efficiency was further decreased in mice fed supplemental uric acid. Animals consuming the 3% plus, and the 5% plus or minus, urate diets exhibited polyuria (Table I). These animals consumed approximately 200% more water than animals receiving the other treatments.

Plasma urate was increased approximately 250% by supplemental oxonate after 7 days of feeding (Table II). Urate supplementation did not significantly influence the effect of KOx during this time. Longer feeding of the urate-supplemented diets accentuated the hyperuricemia.

Creatinine excretion varied considerably, and no significant differences were detectable. Metabolites were expressed per unit of creatinine to normalize the data and provide a more controlled basis for comparison. Urinary uric acid excretions were consistent with plasma urate concentrations (Table II). Oxonate feeding alone increased the excretion of uric acid by 48 and 112% for 3 and 5% KOx, respectively. After the second week of feeding these diets, uric acid excretion was increased only 22 and 20%, respectively. Supplementation with uric acid increased urinary uric acid excretion 36, 205, and 200% in mice fed 1, 3, and 5%, respec-

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TABLE I. EFFECT OF DIETARY OXONATE AND URATE ON GROWTH, FEED INTAKE, AND URINARY EXCRETION IN THE MOUSE.[a]

Dietary supplementation		Growth[b]		Feed intake[c] (g/day)		
Oxonate (%)	Urate (%)	7 days	14 days	7 days	14 days	Urine volume[d] (ml)
0	0	6.6 ± 0.6[1,2]	10.0 ± 0.9[1]	8.9 ± 1.5[1,2]	11.3 ± 1.0[1]	1.4 ± 0.3[1]
0	1	8.0 ± 0.8[1]	10.5 ± 0.5[1]	11.6 ± 0.0[1]	11.8 ± 0.3[1]	1.2 ± 0.4[1]
1	0	4.5 ± 0.6[2]	7.5 ± 1.0[1,2]	10.2 ± 1.5[1,2]	11.0 ± 2.5[1]	2.1 ± 0.2[1,2]
1	1	3.8 ± 0.6[2]	5.0 ± 0.8[2,3]	7.9 ± 0.9[2]	9.6 ± 1.0[1]	1.7 ± 0.3[1]
3	0	7.0 ± 0.8[1,2]	8.7 ± 0.6[1,2]	8.4 ± 0.6[1,2]	10.9 ± 1.1[1]	1.8 ± 0.4[1]
3	1	−0.4 ± 1.9[3]	1.8 ± 3.8[3,4]	2.3 ± 0.4[3]	4.4 ± 4.5[2]	3.8 ± 1.3[2]
5	0	−1.4 ± 1.0[3]	−0.6 ± 0.5[4]	4.0 ± 1.3[3]	8.0 ± 0.3[1]	6.8 ± 2.0[3]
5	1	−3.8 ± 1.2[3]	−4.5 ± 0.5[5]	2.5 ± 0.2[3]	5.2 ± 2.2[2]	3.6 ± 0.8[2]

[a] Mean ± SEM without a common superscript differ, $P < 0.05$. Superscripts are denoted by the numbers 1-5.

[b] Body weight change in grams after 7 and 14 days of experimental feeding.

[c] Grams of diet = 50% dry matter + 50% H_2O.

[d] Day 7 of feeding.

TABLE II. PLASMA CONCENTRATIONS AND DAILY EXCRETION OF URATE IN MICE FED OXONATE WITH OR WITHOUT URIC ACID.[a]

		Plasma		Urinary	
Oxonate (%)	Urate (%)	Day 8 (mg%)	Day 15 (mg%)	Day 7 (μg/μg of creatinine)	Day 14 (μg/μg of creatinine)
0	0	1.8 ± 0.1[1]	2.0 ± 0.3[1]	2.0 ± 0.2[1]	2.3 ± 0.1[1]
0	1	2.9 ± 0.4[1]	4.1 ± 1.0[2]	3.0 ± 0.6[1]	3.6 ± 0.5[1]
1	0	4.9 ± 0.5[2,3]	1.4 ± 0.2[1]	1.7 ± 0.1[1]	2.4 ± 0.1[1]
1	1	6.0 ± 0.9[3]	6.4 ± 0.5[3]	4.0 ± 0.2[1,2]	4.1 ± 1.1[1,2]
3	0	4.7 ± 1.4[2,3]	4.1 ± 0.3[2]	3.0 ± 0.8[1]	2.8 ± 0.2[1]
3	1	4.4 ± 0.5[2,3]	5.1 ± 0.8[3]	9.1 ± 1.4[2]	5.8 ± 2.6[1,2]
5	0	4.1 ± 0.1[2]	3.5 ± 0.3[2]	4.2 ± 0.4[1,2]	2.8 ± 0.3[1]
5	1	4.8 ± 0.3[2,3]	5.7 ± 0.9[3]	8.9 ± 1.4[2]	8.9 ± 1.5[2]

[a] Means ± SEM without a common superscript differ, $P < 0.05$. Superscripts are denoted by the numbers 1-3.

tively. The increase in uricosuria persisted throughout the feeding period. Uric acid excretion was 15, 63, and 119% for the 1, 3, and 5% oxonate plus urate diets, respectively, after 14 days of feeding.

Weights of spleen, kidney, and liver were not significantly altered by any dietary treatment. Neither liver urate or uricase activity were affected significantly by the dietary regimen. However, kidney uric acid increased with increasing oxonate and was further increased by supplemental urate (Table III). One animal consuming the 5% oxonate plus urate diet had grossly visible kidney urate deposits. Kidney uricase activity tended to be depressed by increasing oxonate and further depressed by supplemental urate (Table III).

Urea excretion expressed per gram of N consumed tended to increase with supplemental oxonate and urate after 2 weeks of feeding (Table IV). Urinary ammonia excretion (Table IV) was slightly depressed by oxonate and further depressed by urate supplementation. Orotic acid excretion in mice fed the KOx diets is shown in Table IV. Orotate excretion was increased 10-fold in animals consuming 1% oxonate after Week 2. Further increases were seen with increasing oxonate. Urate supplementation of the diet did not affect orotate excretion. Specificity of the method for orotic acid was checked with large concentrations of oxonate, allantoin, and uric acid. These compounds produced negligible results.

Discussion. Chronic hyperuricemia and renal diseases are classic symptoms of patients with gout. Chronic renal disease has

TABLE III. EFFECT OF DIETARY OXONATE AND URATE ON LIVERS AND KIDNEYS OF MICE.[a]

supple-ation	Organ weights (mg/g body weight)		Uric acid concentration (μg/g of tissue)		
Urate (%)	Liver	Kidney	Liver	Kidney	Uricase[b] Kidney
0	52.2 ± 2.0[1]	15.6 ± 1.0[1]	78.9 ± 14.4[1]	17.8 ± 3.1[1]	66.9 ± 14.8[3]
1	54.8 ± 0.8[1]	16.0 ± 0.9[1]	54.7 ± 4.0[1]	21.1 ± 3.4[1]	40.0 ± 7.3[1, 2, 3]
0	53.3 ± 3.3[1]	15.2 ± 0.8[1]	63.2 ± 14.7[1]	25.3 ± 3.4[1, 2]	39.1 ± 7.2[1, 2, 3]
1	53.3 ± 3.5[1]	17.1 ± 0.6[1]	88.2 ± 20.5[1]	37.5 ± 9.0[1, 2]	28.6 ± 2.8[1]
0	48.0 ± 2.5[1]	17.1 ± 0.8[1]	85.9 ± 18.6[1]	29.2 ± 3.2[1, 2]	51.0 ± 11.9[1, 2, 3]
1	45.3 ± 4.6[1]	16.0 ± 0.4[1]	72.4 ± 12.4[1]	32.1 ± 7.3[1, 2]	30.3 ± 7.7[1, 2]
0	49.7 ± 3.7[1]	15.4 ± 0.7[1]	93.2 ± 21.8[1]	36.1 ± 7.6[1, 2]	31.8 ± 5.2[1, 2]
1	44.8 ± 2.2[1]	15.6 ± 0.4[1]	80.3 ± 17.4[1]	51.0 ± 10.4[2]	61.2 ± 8.1[2, 3]

s ± SEM without a common superscript differ, $P < 0.05$. Superscripts are denoted by the numbers 1-

noles of uric acid per hour per milligram of protein.

TABLE IV. EFFECT OF DIETARY OXONATE AND URATE ON URINARY METABOLITES.[a, b]

y supplementation		Urea-N (mg/gram of N consumed)	NH_3-N (mg/mg of creatinine)	Orotate (μg/mg of creatinine)
(%)	Urate (%)			
	0	432 ± 55[1]	6.7 ± 1.1[1]	304 ± 27[1]
	1	254 ± 44[1]	3.4 ± 0.2[1, 2]	135 ± 16[1]
	0	232 ± 69[1]	2.8 ± 0.4[1, 2]	3,752 ± 1,089[2]
	1	427 ± 73[1]	2.2 ± 0.0[2]	3,589 ± 228[2]
	0	359 ± 77[1]	3.0 ± 0.4[1, 2]	6,617 ± 953[2]
	1	943 ± 78[1]	2.8 ± 0.1[1, 2]	7,653 ± 869[2]
	0	251 ± 10[1]	4.1 ± 1.8[1, 2]	10,347 ± 2,845[2]
	1	580 ± 146[1]	1.8 ± 0.8[2]	9,275 ± 1,921[2]

s ± SEM without a common superscript differ, $P < 0.05$. Superscripts are denoted by the numbers 1

4 of feeding.

erved in approximately 40% of the suffering from gout. Difficulty in a suitable model for gout have l the study of this disease. Recently, m oxonate has proven to be a valu*ivo* tool for examination of hyperurnd nephropathy in the rat (4–6,

have been shown to tolerate rela:ge dosages of KOx for long periods (4, 5, 21, 22). However, the fetus more susceptible to toxic effects of . Gralla and Crelin (11) and Gralla orted that KOx was toxic to the ing fetus when fed to pregnant rats at a dietary concentration of 3%. quantities up to 1 g/day for 28 days resulted in toxicity in the rat althese animals exhibited a hyperurind uricosuria (4). Uric acid supplen accentuates the pharmacological of oxonate (4).

ate alone (5%) dramatically affects feed intake or growth in the mouse. However, neither are appreciably affective in the rat (4, 6). Kox also affects feed efficiency in the mouse. Johnson *et al.* (4) reported a 50% reduction in growth and a 20% reduction in feed intake in rats consuming 5% KOx plus 1% urate diets. The mouse shows a far greater reduction in growth and feed intake at lower dietary concentrations of oxonate plus urate. These data indicate that the mouse may be much more susceptible to KOx than is the rat. The lethality of KOx in the mouse is unknown.

Blood urate concentrations of rats fed oxonate supplemented with uric acid vary considerably (4, 6, 7). Primarily, these variations may be explained by the duration of feeding and the percentages of oxonate and uric acid incorporated into the diets. Gralla and Crelin (11) reported that control nonpregnant female mice receiving chow had mean serum urate concentrations of 3.9

mg%. After 3 days on a chow diet supplemented with 3% KOx, these same animals were found to have a mean serum urate concentration of 7.0 mg%. We observed control mice on a semipurified diet to have a mean serum urate concentration of 2.0 mg% after 2 weeks. Mice consuming 3% KOx had a mean serum urate concentration of 4.1 mg% after the same period. The differences in control serum urate concentrations may be explained by the presence of dietary purines in the chow diets. Oxonate alone appears to increase blood urate concentrations more effectively in the mouse than in the rat.

Urinary uric acid excretion also varies markedly. In this respect, KOx appears to have a greater effect in inducing uricosuria in the rat than in the mouse. Rats show a 600% increase in urinary uric acid excretion with 5% dietary KOx (4). A 20-fold increase in urinary urate was noted in rats consuming 5% KOx plus 1% uric acid supplemented to a chow diet. No significant increase in urinary uric acid excretion after 2 weeks was seen in mice consuming KOx alone, and only a three-fold increase was observed in mice consuming 5% KOx plus 1% urate. The differences between diets may again partially explain these variances. Rats fed a chow diet excrete approximately 2.1 mg of uric acid/24 hr (4). In our experiment the control mice excreted 1.0 mg of uric acid/24 hr.

The study of hyperuricemic nephropathy has possibly been the most significant benefit of the oxonate model. Waisman and his associates (6, 22) concluded that there is a high correlation between the morphology of the nephropathy of oxonic acid-induced hyperuricemia and gout. Urate deposits in the urinary tracts of rats have not been reported if fed oxonate without supplemental urate. However, in several studies, rats fed oxonate supplemented with uric acid developed hyperuricemia with secondary renal effects within 2 weeks (4–6). Feeding mice 5% KOx plus 1% urate for 2 weeks increased kidney uric acid concentrations approximately 300% above animals not consuming oxonate or urate. In the presence of uric acid, KOx induced gross accumulation of urate in only one of the animals. Prolonged feeding probably would have induced deposits in the mouse similar to those previously reported for the rat.

The present experiment shows that dietary KOx has a tremendous effect on pyrimidine metabolism. Previously, Cihak and Sorm (24) showed that mice injected with KOx (5-aza-orotic acid) had decreased utilization of orotic acid for the synthesis of liver nucleic acids which was paralleled by an increased orotic aciduria. *In vitro* studies with extracts from rat and mouse liver indicate that KOx may induce an orotic aciduria by inhibiting orotate phosphoribosyltransferase and ortidylic acid decarboxylase activities (24–26). The present experiment shows that dietary KOx also leads to dramatic alterations in *in vivo* pyrimidine metabolism. These findings may well prove dietary KOx will serve as a useful tool for *in vivo* examination of purine and pyrimidine synthesis, their catabolism, and their interrelationship.

More extensive examination of detoxification of KOx in the mouse vs the rat may help explain the differences between these two animals. Our results indicate the mouse is more sensitive to the toxic effects of oxonate. However, the less severe hyperuricemia and uricosuria suggest a dramatic difference in the handling of KOx in the mouse compared to the rat.

Summary. The effects of dietary supplementation of various concentrations of KOx with or without uric acid were examined in the growing mouse. Many of the effects of KOx were accentuated at lower concentrations when uric acid was also supplemented to the diet. Hyperuricemia was caused by oxonate alone as well as by 1% KOx plus 1% uric acid. Oxonate plus uric acid induced an uricosuria. Kidney uric acid content and uricase activity were significantly altered by oxonate with or without urate supplementation. Oxonate in some ways alters pyrimidine metabolism and leads to an orotic aciduria in the mouse. Oxonate may prove to be a valuable tool in the study of the metabolism of purines and pyrimidines as well as their interrelationship.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 116-120 (1978)

Production of Renal Hypertension in Adrenalectomized Dogs on Constant Hormone Replacement Therapy (40003)

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The onset of renovascular hypertension occurs concurrently with transient activation of the renin-angiotensin system (1-3). Angiotensin II (AII) directly stimulates aldosterone secretion from the adrenal cortex (4-7). Indeed, plasma aldosterone levels and aldosterone secretion rates increase during the acute high renin phase of renovascular hypertension (3, 8, 9). Thus, it is possible that genesis of this pathophysiological condition is dependent on mineralocorticoid hypersecretion.

The present studies were designed to evaluate whether excess mineralocorticoid activity resulting from activation of the renin-angiotensin-aldosterone system is essential for production of sustained renovascular hypertension. For this study an experimental model was used in which increased angiotensin II levels characteristic of the acute high renin phase of renal hypertension could not exert a direct steroidogenic effect on the adrenal cortex. In this regard, the renal artery was constricted in one kidney, adrenalectomized dogs receiving constant exogenous steroid maintenance therapy.

Materials and Methods. Calm female hounds (14.5 to 25.0 kg) were anesthetized with sodium pentobarbital and laparotomized. A unilateral nephrectomy was then performed after verifying the existence of a single renal artery leading to the remaining kidney. Chronic femoral arterial and venous catheters were passed subcutaneously and exteriorized between the shoulder blades in most of these dogs. The dogs were allowed to recover for 7 days after surgery before beginning several days of control data collection. They were fed a daily diet containing 60-65 mequiv of sodium, and water was available *ad libitum*. Urine collections were made for determination of daily urinary output and sodium balance. Arterial pressure was measured from the femoral arterial catheters in conscious animals. Arterial blood samples were also collected and centrifuged for plasma separation and bioassay of plasma renin activity (PRA). Plasma electrolytes were determined by flame photometry and blood hematocrits were measured. In the four dogs without chronic catheters, blood pressure was measured by direct percutaneous femoral artery puncture under local anesthesia, and blood samples were obtained from the jugular vein.

One-kidney renovascular hypertension was produced in seven dogs. The renal artery of the kidney was exposed retroperitoneally. After bathing the area with 2% lidocaine solution, an adjustable constricting device and an electromagnetic flow probe were placed around the renal artery. After allowing 10-20 min for stabilization of blood flow, the renal artery was constricted to reduce renal blood flow by 56 ± 2%. After renal artery constriction, data were obtained daily for 7 days and, thereafter, three times each week for at least 1 month; chronic sodium balance studies were also conducted. The dogs were force fed when necessary to assure constant sodium intake. Prior to and at weekly intervals after renal artery constriction, plasma volume was also estimated by T-1824 dye dilution.

An additional group of eight dogs underwent bilateral adrenalectomy when the unilateral nephrectomy was performed. These dogs received pretreatment with 100 mg of cortisone acetate im the day before surgery. On the morning of the day of surgery, they were given 100 mg of cortisone orally, and an additional 50 mg of cortisone im immediately before adrenalectomy. Thereafter, the dogs were maintained on daily im ste-

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apy of 20 mg of cortisone acetate
ıg of deoxycorticosterone acetate
, and data were collected immedi-
ore steroid therapy, steroids were
a single dose at 9:30 AM. The
ery was constricted 1 week after
ctomy. The first four animals of
ıp were given a supplemental dose
ıg of cortisone acetate im immedi-
er renal artery constriction. Since
mals recovered readily, the remain-
adrenalectomized dogs received no
ıl cortisone during the surgery to
the renal artery. Observations
de in the same manner as for the
dogs with intact adrenals. Com-
ı of adrenalectomy was verified by
o measure discernible levels of
ıldosterone by a radioimmunologi-
ique (7).

rom all experiments were analyzed
f the Student's paired *t* test or by
ı *t* test for group comparisons, crit-
5% significance level.

:. *Renal artery constriction in dogs
ıct adrenals.* Constriction of the
ery in the seven dogs having intact
produced a prompt increase in
ıressure in all but one animal. The
le clip was later retightened in this
renal hypertension occurred. Mean
ıressure increased during the first
r renal artery constriction in all
< 0.05) and reached a steady level
ion of approximately 20 mm Hg by
nd or third day after constriction
. Studies were terminated in two
hin 2 weeks after renal artery con-
because they developed malignant
sion.

gs with intact adrenals, PRA in-
:ransiently ($P < 0.05$) for the first
ıfter renal artery constriction and
urned to control levels (Fig. 2).
ative sodium balance data for this
howed that sodium retention of
ı mequiv occurred during the first
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reted most of this excess salt load
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er, they generally remained in nor-
um balance, although chronically
ıy have been a tendency toward

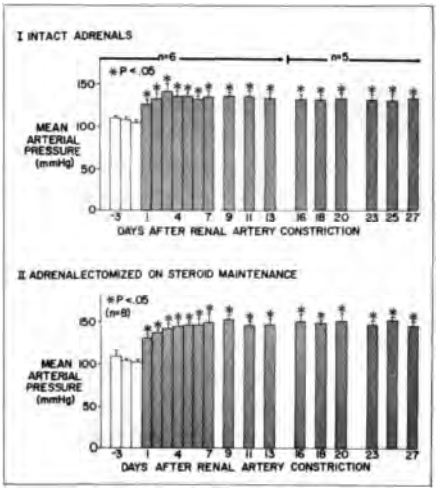

FIG. 1. Changes in mean arterial pressure after renal artery constriction in dogs with intact adrenals and in adrenalectomized dogs on constant steroid maintenance of cortisone acetate (20 mg/day) and DOCA (2 mg/day). Asterisks denote differences significant at 5% level from corresponding preconstriction control values, and shaded bars indicate values after renal artery constriction. Data appear as the mean ± SEM.

slight sodium retention. However, there was no detectable expansion of plasma volume during the 4 weeks after renal artery constriction. Hematocrit decreased from 36 ± 2 to 28 ± 2 ($P < 0.05$) during the first 6 days after renal artery constriction and then returned toward control levels.

Renal artery constriction in adrenalectomized dogs. Hypertension occurred following the initial constriction of the renal artery in six of the eight adrenalectomized dogs. Reconstricting the renal artery successfully produced sustained hypertension in the other two dogs. Apparently, hypertension was equally well produced in adrenalectomized dogs whether or not they received supplemental cortisone acetate therapy immediately following constriction of the renal artery. Also, since supplemental cortisone did not in itself affect any other variables that were measured, the data were pooled for all eight of these adrenalectomized dogs.

Mean arterial pressure rose promptly after renal artery constriction ($P < 0.05$) (Fig. 1), and the response was not different from that observed in dogs with intact adrenals. The adrenalectomized animals also

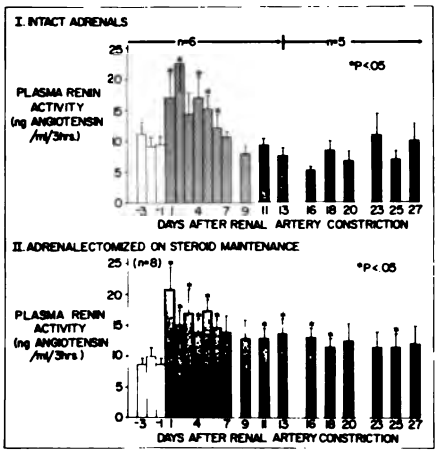

FIG. 2. Changes in plasma renin activity after renal artery constriction in dogs with intact adrenals and in adrenalectomized dogs on constant steroid maintenance of cortisone acetate (20 mg/day) and DOCA (2 mg/day). Asterisks denote differences significant at 5% level from corresponding preconstriction control values, and shaded bars indicate values after renal artery constriction. Data appear as the mean ±SEM.

showed a transient decrease in hematocrit after constricting the renal artery, similar to that which occurred in intact dogs. Further, they exhibited a marked increase in PRA for 6 days of approximately the same magnitude as occurred in the dogs with intact adrenals (Fig. 2). However, PRA levels remained somewhat elevated throughout the observation period in the adrenalectomized group, a response different from that of the dogs with intact adrenals. Renal artery constriction produced virtually the same pattern of changes in cumulative sodium balance in the adrenalectomized dogs as occurred in the dogs with intact adrenals with one exception: Sodium balance was slightly positive during the last week in the adrenalectomized dogs. However, after production of renal hypertension in the adrenalectomized dogs, plasma volume increased from a control value of 1040 ± 90 to 1205 ± 50, 1165 ± 35, and 1335 ± 105 ml for the next 3 weeks ($P < 0.05$ for all three values), so the additional sodium retention during the fourth week did not manifest itself by an increase in plasma volume.

The results also show that the daily steroid maintenance of 2 mg of DOCA and 20 mg of cortisone acetate was adequate to maintain normal plasma electrolyte concentrations. In dogs with intact adrenals, control plasma sodium and potassium concentrations were 143 ± 5 and 4.3 ± 0.1 mequiv/liter, respectively, while corresponding values in adrenalectomized dogs before renal artery constriction were 144 ± 1 and 4.2 ± 0.2 mequiv/liter. Production of renal hypertension did not alter these plasma electrolyte levels in either group.

Discussion. Early investigations on the role of the adrenal cortex in the genesis of renal hypertension were conducted 40 years ago (10, 11). Much of the existing literature suggests that induction of renal hypertension requires adequate adrenocortical activation. However, it has been uncertain whether the general inability to produce or sustain renal hypertension in nonmaintained, adrenalectomized animals is due to a nonspecific effect of adrenal origin (12-14). A possible adrenal factor which has been considered necessary for induction of renal hypertension is mineralocorticoid hypersecretion. This is an attractive possibility since the renin-angiotensin-aldosterone system is known to be activated during the early phase of renal hypertension (3, 8). In fact, Atwill *et al.* (15) reported that renal hypertension could not be induced or maintained in adrenalectomized dogs receiving only glucocorticoid replacement therapy. Also, it has been reported that spironolactone, a competitive inhibitor of aldosterone, reverses chronic renal hypertension in the rabbit (16). On the other hand, there is considerable evidence that aldosterone hypersecretion may not be necessary. Renal hypertension was produced despite administration of an aldosterone antagonist (17) and was maintained in adrenalectomized sheep for 48 hr after withdrawal of steroid therapy (18).

The purpose of the present study was to determine if chronic hypertension would develop in adrenalectomized dogs following renal artery constriction during maintenance on constant steroid replacement therapy. Under these circumstances the animals were never subjected to a high plasma level of aldosterone or mineralocorticoid excess for even a brief period. The experiment was done to exclude the possibility that

ocorticoid excess for a short time rigger a pathogenic mechanism im- for the development of chronic hy- ion. Available data have never an- the question of the necessity of early on of the renin-angiotensin-aldos- system for chronic hypertension to The present results were clearly neg- :hronic hypertension developed and stained for 27 days during a constant se of 2 mg/day of DOCA along with of cortisone daily. This dose was d because in our past experience (19) evels of replacement therapy are no than enough to keep adrenalecto- dogs healthy.

results of the current study agree e already well-documented literature ning the pathogenesis of one-kidney scular hypertension. Specifically, the ngiotensin system is activated during t few days in this form of hyperten- has been suggested (1, 2) that this hase is largely renin dependent, but one-kidney hypertension is clearly ndependent; the present dogs with drenals had normal values for PRA chronic hypertension. Also, the cu- e sodium balance data do not pro- idence for further extracellular fluid expansion during the chronic phase ertension. Our findings support re- ports (20, 21) that one-kidney renal ension is sustained by factors other pervolemia per se.

ntrast to the dogs with intact adre- he chronic adrenalectomized hyper- dogs had a significant elevation in This finding indicates that the renin- nsin system might contribute to the maintenance of hypertension in this lectomized model, as has been re- previously (22). The stimulus for onic elevation of PRA in these adre- mized hypertensive dogs is un- . These animals were not in negative balance during the last 2 weeks; sodium balance was slightly positive the last week. Also, plasma volume reased and plasma electrolytes were , so there is no obvious explanation increase in renin release.

nary. This study assessed the impor- tance of increased aldosterone secretion secondary to acute activation of the renin–angiotensin system for development of chronic one-kidney renal hypertension. In dogs with intact adrenals, reduction of renal blood flow by 55–60% promptly produced mild chronic hypertension. The onset of hypertension was associated with transient sodium retention and hyperreninemia, but plasma volume did not change. Although mineralocorticoid hypersecretion was impossible in adrenalectomized dogs maintained with constant steroid therapy, hypertension was still successfully produced. In contrast to the dogs with intact adrenals, hypertension in the adrenalectomized group was characterized by chronically increased plasma renin activity and plasma volume. The results indicate that hypersecretion of mineralocorticoid hormones during the early high renin phase is not essential for production of chronic one-kidney renal hypertension.

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)F THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 121-124 (1978)

ion of Angiotensin-Converting Enzyme by SQ 14,225 in Anesthetized Dogs: Hemodynamic and Renal Vascular Effects (40004)

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tion of vasomotor and endocrine of the renin-angiotensin system by ing renin release, preventing con- of angiotensin I (AI) to angiotensin , or blocking the receptor sites for h specific antagonists has received able attention for the diagnostic rapeutic management of hyperten- ases. Competitive inhibition of an- n-converting enzyme (ACE) by a tide, SQ 20,881, has offered a proach to the treatment of hyper- (1-3). However, the therapeutic l of SQ 20,881 is limited by its lack activity. Recently Ondetti and co- (4) have reported the development 4,225 (D-3-mercapto-2-methylpro- -proline), an orally active competi- bitor of ACE.

is investigation, the hemodynamic al vascular effects of inhibition of ith SQ 14,225 were evaluated in ized dogs.

ds. Mongrel dogs weighing 10 to ere anesthetized with sodium pen- al (30 mg/kg iv). After endotracheal on, artificial respiration was insti- ing a Harvard respirator. The right vein was cannulated for intravenous is, and the right femoral artery was ted for the recording of arterial pres- ie heart rate was recorded by trig- a cardiotachometer with arterial pulse. A catheter-tip pressure er (Mikro tip, Millar Instruments, uston, Texas) was introduced into ventricle through the left common rtery, and the left ventricular pres- recorded. The rate of rise of left lar pressure (*dP/dt*) was obtained onic differentiation of the left ven- tricular pressure pulse. The left kidney was exposed using a flank incision and a precalibrated electromagnetic flow probe of suitable size was positioned on the left renal artery. The flow signal was recorded using a Biotronex flowmeter. The ureters were cannulated for timed collection of urine. All signals were recorded on an eight-channel Beckman Dynograph Recorder.

The dogs were divided into four groups. In the first group, consisting of five dogs, effects of increasing doses of SQ 14,225 on the pressor and renal vasoconstrictor effects of AI (310 ng/kg) and AII (100 ng/kg) were investigated. After obtaining control responses to AI and AII, SQ 14,225 was administered iv in half-log dose increments (10 to 310 μg/kg) at 30-min intervals and after each dose the responses to AI and AII were studied. In the second group of four dogs, the duration of ACE inhibition after 310 μg/kg iv of SQ 14,225 was investigated. Responses to AI were studied before and at 10-min intervals after an iv dose of 310 μg/kg of SQ 14,225 for 120 min. In the third group of eight dogs, the cardiovascular and renal effects of a single iv dose of 310 μg/kg of SQ 14,225 were investigated. In the last group of four dogs, cardiovascular and renal effects of 5 ml of saline (solvent) were studied. Also the effects of the solvent on the responses to AI and AII were investigated in this group. AI (Asp^1-Ile^5 angiotensin I) and AII (Asp^1-Ile^5 angiotensin II) were injected iv as a bolus while SQ 14,225 was dissolved in 5 ml of saline and infused over a 5-min period. AI and AII were purchased from Schwarz/Mann (Orangeburg, N.Y.). At the end of the experiment the kidney was removed and weighed.

The results are presented as the mean ± SE in the text, table, and figures. Differ-

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ences were considered significant at a level of $P < 0.05$, using Student's t test for paired data.

Results. The resting renal blood flow was 4.1 ± 0.3 ml/min/g of kidney. Intravenous injection of AI and AII caused comparable increases in mean arterial pressure, decreases in renal blood flow, and increases in renal vascular resistance (Fig. 1). After administration of SQ 14,225, a dose-dependent inhibition of the pressor as well as renal vasoconstrictor effects of AI was seen, while saline had no effect (Fig. 2). The renal vasoconstrictor effects were more responsive ($ID_{50} < 10$ μg/kg) to inhibition than the pressor effects ($ID_{50} = 37$ μg/kg). Maximal inhibition of the pressor and the vasoconstrictor effects were seen after 100 and 310 μg/kg of SQ 14,225. The pressor responses to AII were not effected by SQ 14,225 while the vasoconstrictor effects were enhanced (Fig. 2). There was marked variation in the magnitude of potentiation of the vasoconstrictor effects of AII in the individual animals. The animals treated with saline showed no changes in the pressor and vasoconstrictor effects of AII.

Figure 3 illustrates the duration of inhibition of AI-induced pressor and renal vasoconstrictor responses after 310 μg/kg iv of SQ 14,225. Within 10 min of administration of SQ 14,225 the pressor response to AI was inhibited 78%, while 94% inhibition of the renal vasoconstrictor effect was observed. The responses to AI recovered gradually, reaching 50% of the initial response within 60–70 min.

The hemodynamic and renal vascular effects of a single dose of SQ 14,225 (310 μg/kg iv) are presented in Table I. The ACE inhibitor caused a moderate decrease in arterial pressure within 10 min, and complete recovery was seen within 40 min. At the peak of hypotensive effect, a small increase in the heart rate was seen. The left ventricular dP/dt max remained unchanged. After SQ 14,225, renal blood flow in-

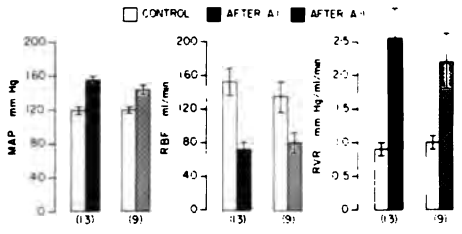

FIG. 1. Effects of angiotensin I (AI, 310 ng/kg, iv) and angiotensin II (AII, 100 ng/kg iv) on mean arterial pressure (MAP), renal blood flow (RBF), and renal vascular resistance (RVR) in anesthetized dogs. Numbers in parentheses equal number of animals.

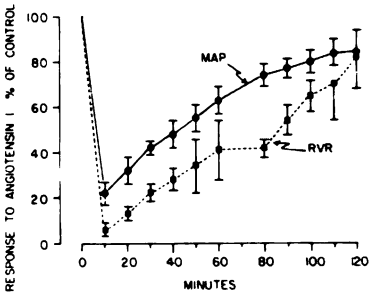

FIG. 3. Temporal changes in the magnitude of angiotensin I (AI, 310 ng/kg iv) induced alterations in mean arterial pressure (MAP) and renal vascular resistance (RVR) after intravenous administration of SQ 14,225 (310 μg/kg) in anesthetized dogs.

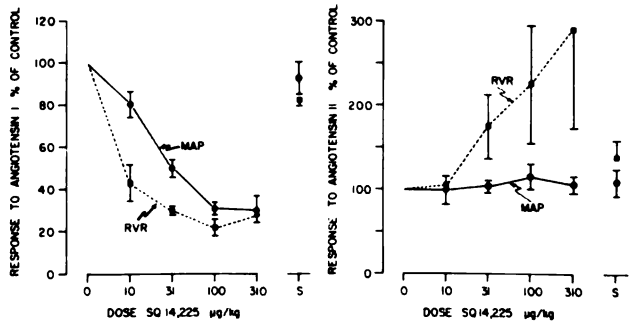

FIG. 2. Effects of intravenously administered SQ 14,225 and saline (s) on the pressor and renal vasoconstrictor effects of angiotensin I (AI, 310 ng/kg iv) and angiotensin II (AII, 100 ng/kg iv) in anesthetized dogs. MAP, Mean arterial pressure; RVR, renal vascular resistance.

TABLE I. HEMODYNAMIC AND RENAL VASCULAR EFFECTS OF SQ 14,225.[a]

	SQ 14,225 (n = 8)[b]		Saline (n = 4)[b]	
	Control	10 min[c]	Control	10 min[c]
' (mm Hg)	152 ± 5	139 ± 5[d]	146 ± 7	150 ± 7
iP (mm Hg)	114 ± 4	96 ± 5[d]	110 ± 3	113 ± 3
(beats/min)	145 ± 6	150 ± 7[d]	162 ± 13	157 ± 14
ricular *dP/dt* max /sec)	2672 ± 191	2757 ± 215	2811 ± 299	2815 ± 340
id flow (ml/min)	185 ± 11	205 ± 14[d]	115 ± 16	118 ± 17
ular resistance (mm iin)	0.70 ± 0.03	0.55 ± 0.04[d]	1.13 ± 0.19	1.14 ± 0.19
ut (ml/10 min)	2.26 ± 0.33	2.17 ± 0.38	1.62 ± 0.44	1.54 ± 0.38

1,225 dose: 310.0 μg/kg iv.
lumber of dogs used in the test.
inutes after infusion of SQ 14,225 or saline.
.05.

by 11% above control value and return to control level up to 60 ie renal vascular resistance de- Urine output remained unchanged. ous administration of saline had no n any of the parameters studied.

sion. The release of renin from the regulates the vasomotor and endo- luences of the renin-angiotensin- one axis. The ACE, a peptidyldi- hydrolase, converts the biologically AI to AII, the most potent endog- vasoconstrictor known (5). SQ as been found to be a potent com- inhibitor (K_i = 1.7 × 10^{-9} M) of it lung ACE (4, 6). In isolated ig ileum, intact rat (7), and anesthe- (8), it inhibited the effects of AI those induced by AII. Our results greement with those of the above tors. In addition, we found en- nt of the renal vasoconstrictor ef- AII after SQ 14,225. Since inhibi- ACE would result in loss of endoge- I, enhancement of the effects of ld be due to expansion of free population for exogenous AII as suggested by Thurston and Laragh

r experiments with anesthetized 14,225 decreased arterial pressure ed renal vasodilation. In conscious bition of ACE with SQ 20,881 has nd to have no effect on renal blood , 11). However, in anesthetized al vasodilation was observed by vestigators (10, 12-14). It appears that renal vascular effects of ACE inhibitors as well as AII receptor antagonists depend upon the level of activity of the renin-angiotensin system. In dogs, barbiturate anesthesia activates the renin-angiotensin system (10, 15, 16) and reduces renal blood flow (10). Furthermore, the renin-angiotensin system is activated in dogs subjected to negative sodium balance. In sodium-depleted dogs either ACE inhibition with SQ 20,881 or AII receptor blockade with saralasin (1-Sar, 8-Ala angiotensin II) causes renal vasodilation (10, 11, 17). It is therefore proposed that in dogs under barbiturate anesthesia endogenously generated AII contributes significantly to resting renal vascular resistance, and inhibition of ACE with SQ 14,225 removes this vasoconstrictor component and causes vasodilation. However, DiSalvo and co-workers did not observe any change in renal blood flow after ACE inhibition with the pentapeptide inhibitor SQ 20,475 (BPP 5a) in anesthetized dogs (18). The reasons for this descrepancy are not understood presently. The dogs which received saline had lower resting renal blood flow and urine output compared to the group receiving the drug (Table I). Since in both treatments the dogs served as their own controls, it is unlikely that this difference could have influenced the results and the conclusions of this study. The decrease in arterial pressure seen in our experiments after SQ 14,225 could also be due to loss of endogenous AII and the associated transient tachycardia, a reflex-mediated response.

Rubin and co-workers have shown that SQ 14,225 enhances the hypotensive effects of bradykinin (7), since ACE plays an important role in the inactivation of bradykinin (19). It is therefore possible that endogenously generated bradykinin may also contribute to the renal vasodilation seen after SQ 14,225. Although Miller and co-workers (20) failed to demonstrate any increase in circulating levels of bradykinin after inhibition of ACE with SQ 20,881 in dogs, the role of kinins of renal origin cannot be excluded (14).

Contrary to our observations with SQ 14,225, urine output was found to increase after ACE inhibition with SQ 20,881 (12, 14). This disparity could be due to the state of water balance of the animals, since in both the above studies a continuous saline infusion was maintained.

Summary. The hemodynamic and renal vascular effects of SQ 14,225, a new angiotensin-converting enzyme inhibitor, were studied in mongrel dogs under pentobarbital anesthesia. A dose-related inhibition of the pressor and renal vasoconstrictor effects of AI, but not of AII, were seen after SQ 14,225. After administration of SQ 14,225 (310 μg/kg iv) a moderate decrease in arterial pressure and increase in renal blood flow were observed. It is proposed that the hypotension and renal vasodilation seen after SQ 14,225 could be due to loss of endogenous AII; however, an enhancement of endogenously produced bradykinin cannot be excluded.

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# of Sindbis Virus Infection on Hydrocortisone-Induced Hepatic Enzymes in Mice (40005)

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ɛron and interferon-inducing agents *vivo* and *in vitro* conditions have nd to cause effects which are apparrelated to interferon's well-known o inhibit viral replication. These altered immune responses (1-3), l replication of neoplastic cells (4), d rate of liver regeneration (5, 6), for neonatal mice (7), and sensitif adult mice (8, 9) and rats (10) to gative bacterial endotoxin. Renton ınering (11) demonstrated that an of interferon-inducing agents in ressed hepatic cytochrome *P*-450 ıd the activities of cytochrome *P*-ɛndent monooxygenases. Other inɪrs suggest that interferon is also inhibit the hormonal induction of ıepatic enzymes (12, 13).

ave shown that administration of endotoxin (14, 15) or double-polyribonucleotide [poly(rI)·] (9) to mice results in inhibited ıl induction of hepatic phosphoıvate carboxykinase (EC 4.1.1.32). ıdotoxin and poly(rI)·poly(rC) are ɛrferon inducers (16, 17), the poshat viral infection also inhibits enduction was investigated. Results in this communication show that urs in mice infected with Sindbis ı arbovirus that produces a nonlection in adult mice (18).

ials and methods. Pathogen-free, 5-ɛk old, female Ha/ICR mice were these studies and were maintained 12-hr light-dark cycle. Unless oth-ɪated, food (Wayne Lab Blox) and ɛre available at all times. Sindbis eparations (ATCC strain A339) were prepared and titrated on chick embryo fibroblasts and stored at −70°. Activities of hepatic phosphoenolpyruvate carboxykinase (19), tryptophan oxygenase (EC 1.13.11.11) (20), and tyrosine aminotransferase (EC 2.6.1.5) (21) were determined in whole liver homogenates as described previously. Inteferon titers in mouse serum were determined by the 50% plaque reduction assay on mouse L929 cells challenged with vesicular stomatitis virus. Commercial interferon and mock interferon were purchased from Bionetics Laboratory Products (Kensington, Md.). This was produced by infecting C243-3 cells with Newcastle disease virus (NDV) and contained 10^6 units of interferon/ml. Mock interferon, prepared from the same cells, had NDV added after harvest. The virus was inactivated by exposure to pH 2 for 5 days. Statistical significance was determined by the Wilcoxon rank sum test (22).

Results. Initial experiments demonstrated that mice infected intravenously with 1×10^9 plaque-forming units of Sindbis virus maintained a normal base level of phosphoenolpyruvate carboxykinase activity during the first 12 hr (see Table III). The same level of infection suppressed, however, the induction of the enzyme elicited by overnight fasting. These results are shown in Table I. The increase in enzyme activity that occurred in fasted mice was significantly reduced in infected mice. Enzyme induction was also significantly inhibited, in mice injected with an equal volume of Sindbis virus suspension that had been irradiated with ultraviolet light. Since less than 200 plaque-forming units remained following irradiation, these results suggest that viral inhibition of phosphoenolpyruvate carboxykinase (PEPCK) induction is not a direct result of an active viral infection.

The interval between Sindbis injection and the time when hormonal induction of

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phosphoenolpyruvate carboxykinase was inhibited was determined in the same manner as that described previously for poly(rI)·poly(rC) (9). At timed intervals following virus or saline injection, 1 mg of hydrocortisone acetate (Sigma) was injected subcutaneously, and enzyme activity was determined 4 hr later. Results are shown in Table II. Enzyme activity was as fully inducible in virus-infected mice as in normal mice for at least 4 hr. Six hours after virus infection, however, the enzyme was significantly less responsive to hormonal induction ($P \leq 0.001$) and the same was evident also at 8 hr. The total elapsed times were, respectively, 10 and 12 hr.

The titer of interferon was determined in pooled sera of mice 8 hr after an injection of either live virus or irradiated virus in amounts similar to those used to obtain the data in Table I. For live virus the tite 1:160 and for irradiated virus it was 1 Since live virus inhibited the inducti the enzyme significantly more than virus, there is some doubt as to wheth factor responsible for this effect is feron.

This doubt is strengthened by resul tained with a group of mice injected i 25,000 units of commercial interfero tained in 0.2 ml and with another gr animals given an equal volume of interferon. After 8 hr half the mice i group were injected sc with 1 mg of l cortisone acetate and 4 hr later the li all animals were assayed for PEPCK ity. The control and induced values fo given interferon were, respectively, 5 for seven mice and 150 ± 9 for mice. Corresponding values for mice mock interferon were, respectively, 12 for seven mice and 154 ± 10 for mice. It would appear, therefore, tl ruses present in both preparations responsible for inhibiting PEPCK indu This is borne out by the results ob when the interferon and mock inte preparations were centrifuged for 1 105,000*g* and the experiments ju scribed were repeated with the super fluids. The control and induced valu mice given an injection with the fluic the interferon preparation were, r tively, 160 ± 13 for seven mice and 16 for seven mice. Corresponding for mice given injections from the interferon were, respectively, 158 ±

TABLE I. HEPATIC PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY IN FASTED, SINDBIS-INFECTED MICE[a]

Treatment	Number of mice	Enzyme activity[b] (mean ± SEM)	Significance vs fasted control
Fed controls	6	118 ± 15	$P \leq 0.01$
Fasted controls	6	191 ± 9	–
Fasted + 1 × 10^9 Sindbis PFU	6	144 ± 10	$P \leq 0.01$
Fasted + uv-irradiated Sindbis	6	163 ± 8	$P \leq 0.05$

[a] All injections were made iv in 0.1 ml at 5:00 PM when food was withdrawn. Enzyme activity was determined at 8:00 AM the following day.

[b] Micromoles of phosphoenolpyruvate formed in 6 min per gram (dry wt) of liver.

TABLE II. HEPATIC PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY IN NORMAL MICE AND IN M AT DIFFERENT TIMES POSTINFECTION WITH SINDBIS VIRUS[a]

Time of hydrocortisone administration (hr after Sindbis infection)	Enzyme activity[b] (mean ± SEM)			
	Control mice		Mice infected with Sindbis virus	
	Uninduced	Induced[c]	Uninduced	Induced
0	144 ± 7 (8)[d]	249 ± 6 (14)	145 ± 6 (8)	249 ± 12 (4
2	151 ± 10 (8)	249 ± 6 (14)	149 ± 7 (8)	276 ± 19 (1
4	165 ± 11 (8)	262 ± 9 (14)	170 ± 12 (8)	268 ± 12 (1
6	168 ± 12 (7)	292 ± 14 (8)	158 ± 10 (8)	166 ± 7 (7)
8	166 ± 9 (8)	272 ± 8 (7)	151 ± 6 (8)	152 ± 5 (1

[a] Mice were infected iv with 10^9 plaque-forming units of Sindbis virus at time zero. Control mice w infected.

[b] Micromoles of phosphoenolpyruvate formed in 6 min per gram (dry wt) of liver.

[c] Mice were injected sc with 1 mg of hydrocortisone acetate at the time postinfection indicated and th sacrificed for enzyme assays 4 hr later.

[d] Numbers in parentheses represent number of mice used for the value shown.

ce and 281 ± 15 for seven mice. It
kely, therefore, that interferon is
ole for blocking PEPCK induction
experiments.

ffect of Sindbis virus infection on
er corticosteroid-inducible hepatic
was investigated also. Results are
n Table III. Tyrosine aminotrans-
ehaved in a manner similar to that
hoenolpyruvate carboxykinase. Al-
infection did not suppress enzyme
in fed mice, enzyme induction by
rtisone was inhibited significantly in
mice and in mice injected with
rticles which had been inactivated
sure to ultraviolet light.

ffect of Sindbis on tryptophan oxy-
however, differed from that of the
vo enzymes. Although virus infec-
pressed hormonal induction the
el activity of tryptophan oxygenase
ificantly depressed in infected mice.
han oxygenase, unlike the other two
, is apparently sensitive to some
ction of an active viral infection.
gestion is supported by the obser-
hat the enzyme was not only fully
e, but was also superinducible in
jected with inactivated virus parti-
ere is no explanation as to why this

ssion. These results demonstrate
injection of Sindbis virus inhibits
al induction of hepatic phospho-
uvate carboxykinase, tyrosine ami-
notransferase, and tryptophan oxygenase. Bacterial endotoxin (14, 15, 20, 23) and poly(rI)·poly(rC) (9, 24) also inhibit corticosteroid induction of hepatic phosphoenolpyruvate carboxykinase and tryptophan oxygenase. The two toxic substances, however, induce tyrosine aminotransferase activity in mice rather than inhibit its induction (24, 25). Since the enzyme does not induce in endotoxin-treated, adrenalectomized mice (25), the difference between the action of the virus and these two substances is probably dependent on their ability to stimulate adrenal-cortical secretion. Our results suggest that virus injection, unlike endotoxin and poly(rI)·poly(rC), does not result in *in vivo* steroidogenesis.

We have previously shown that the inhibitory effect exerted by endotoxin on phosphoenolpyruvate carboxykinase is mediated by a factor produced by the poisoned host (26, 27) and suggest that inhibited hormonal induction of the three hepatic enzymes (with the possible exception of tryptophan oxygenase) in Sindbis-infected mice is mediated also.

While interferon may be responsible for the inhibitory effects in mice treated with each of these agents, proof will require more highly purified preparations and assay of the enzymes in isolated hepatocytes or a hepatoma cell line. It is important to note, however, that the time required for inhibition of phosphoenolpyruvate carboxykinase induction in Sindbis-infected mice (6 to 10

.E III. Activities of Corticosteroid-Inducible Hepatic Enzymes in Sindbis Virus-Infected Mice[a]

Treatment	Phosphoenolpyruvate carboxykinase[b]	Tyrosine aminotransferase[c]	Tryptophan oxygenase[d]
	148 ± 17 (10)	6.6 ± 0.1 (7)	6.1 ± 0.7 (12)
1 × 10^9 Sindbis PFU	143 ± 10 (5) w	6.0 ± 0.4 (7)	1.8 ± 1.3 (5)[e]
l mg of hydrocortisone	242 ± 10 (6) w	25.6 ± 0.7 (21)	23.3 ± 1.4 (12)
1 × 10^9 Sindbis PFU; 4 hr g of hydrocortisone	196 ± 11 (6)[e]	16.7 ± 0.7 (14)[f]	13.0 ± 1.5 (12)[f]
uv-Sindbis; 4 hr after 1 mg of tisone	183 ± 9 (7)[e]	19.9 ± 0.5 (7)[f]	32.1 ± 1.4 (12)[f]

s virus and uv-inactivated Sindbis were injected intravenously in 0.1 ml of physiological saline at 12:00
Corticosteroid (1 mg of hydrocortisone acetate) was injected subcutaneously in 0.2 ml of saline 8 hr
0 AM. Enzyme activities were determined 4 hr later at 12:00 noon. Values are the mean ± SE, and parentheses represent the number of mice used for each determination.

noles of phosphoenolpyruvate formed in 6 min per gram (dry wt) of liver.

rams of *p*-hydroxyphenylpyruvic acid formed in 10 min per milligram (dry wt) of liver.

noles of kynurenine formed in 1 hr per gram (dry wt) of liver.

antly different from corresponding control ($P \leq 0.01$).

antly different from corresponding control ($P \leq 0.001$).

hr) coincides with the appearance of significant levels of interferon in the serum of virus-infected animals (16). In addition, interferon, at least in relatively high concentrations, is known to inhibit steroid induction of tyrosine aminotransferase in cultured hepatoma cells (12, 28). Our observations with commercially available interferon raise doubt as to whether the above referenced observations and our own in this report are, in fact, due to the action of interferon. This question will remain unsettled until further studies have been completed.

The metabolic significance of inhibited enzyme induction in Sindbis infection remains to be determined. It is pertinent to note, however, that seven fasted, Sindbis-infected mice were significantly more hypoglycemic after 15 hr than an equal number of fasted controls (64 ± 6 versus 87 ± 19 mg of glucose/dl of blood, respectively, $P < 0.05$). While it is premature to attribute the low blood sugar level to a suppressed activity of phosphoenolpyruvate carboxykinase, more careful investigation of carbohydrate metabolism in virus-infected mice may prove revealing.

Summary. Hormonal induction of three hepatic enzymes, phosphoenolpyruvate carboxykinase, tryptophan oxygenase, and tyrosine aminotransferase, was significantly inhibited in mice infected with Sindbis virus. Induction of phosphoenolpyruvate carboxykinase was suppressed whether it was elicited by overnight fasting or by administration of hydrocortisone. This enzyme responded normally to steroid induction 4 hr after virus administration, but after 6 hr, it was significantly less responsive. The timing of this effect corresponds with the appearance of interferon in the serum of virus-infected mice and mice injected with uv-irradiated virus, but results with commercially available interferon make it questionable whether interferon is the factor responsible.

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hibition of Eosinophilotaxis by Chloroquine and Corticosteroids[1] (40006)

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presence of eosinophils is a hallmark gic inflammatory reactions, but the ıyed by these cells in the inflamma- ocess has not been definitely estab- Corticosteroids markedly ameliorate natory reactions in allergic diseases, mechanism of the beneficial effects drugs in these allergic diseases is : (1). Corticosteroids alter the re- ; of certain cells involved in inflam- processes, for example, *in vitro* they te macrophage migration (2, 3), in- onchoalveolar macrophage aggrega-), inhibit the migration of polymor- lear neutrophils (5), and may influ- mphocyte migration (6). The eosin- response induced by corticosteroids has been known for many years, but knowledge no effects of corticoste- ı eosinophils *in vitro* have been dem- ed. Chloroquine hydrochloride, an- anti-inflammatory agent, has also own to affect migration of polymor- lear neutrophils (5), but its action osinophils has not been investigated. study we have assessed the effect of ty of agents including chloroquine ticosteroids on the migration of eo- ls using an *in vitro* chemotactic assay.

rials and methods. Drugs. Hydrocor- odium succinate and methylpredni- odium succinate (Upjohn Co., Kala- Mich.) and chloroquine hydrochlo- gma Chemical Co., St. Louis, Mo.) iluted in: $CaCl_2$, 0.20 g/liter; KCl, liter; $MgCl_2$, 0.0469 g/liter; NaCl, iter; $NaH_2PO_4 \cdot H_2O$, 0.050 g/liter; , 1.0 g/liter (Tyrode's solution, Island Biological Co., Grand Island, which also contained 2% human se- bumin (Sigma Chemical Co., St. Louis, Mo.) and was denoted T-HSA. Estrone and estradiol (Sigma Chemical Co.) were dissolved in absolute ethanol and diluted in T-HSA so that the final ethanol concentration in the cell suspension was 0.5%. The final concentrations of these drugs were 0.02 (7.9×10^{-5} *M*) and 0.1 mg/ml (3.7×10^{-4} *M*), respectively. Testosterone (Sigma Chemical Co.) was dissolved in absolute ethanol to a concentration of 10^{-1} *M* and was diluted with T-HSA to a final concentration of 0.28 mg/ml (10^{-3} *M*) and a final ethanol concentration of 0.5%.

Chemotactants. Two chemotactants were used: eosinophil chemotactic factor of anaphylaxis (ECF-A) and C5a. ECF-A was prepared from human lung tissue according to Wasserman and associates (7). Briefly, normal lung tissue was obtained from patients undergoing pneumonectomy. The pleura, large vessels, and bronchi were dissected off; the tissue was minced with fine scissors and washed in Tyrode's solution until free of blood. It was then suspended in 0.15 *M* NaCl in a proportion of 1 g of wet tissue per 3 ml of saline, homogenized (Virtis homogenizer, Gardiner, N.Y. Model No. 60K) at 15,000 rpm for 1 min and centrifuged at 400*g* for 10 min. Part of the supernatant was stored in small aliquots at −70° and the remainder was lyophilized. Samples were reconstituted in distilled water and analyzed by gel filtration on a Sephadex G-25 column (50 × 1.5 cm) equilibrated with Tyrode's solution. C5a was prepared by treating guinea pig sera with zymosan (Sigma Chemical Co.) according to Kay and associates (8). Briefly, zymosan was boiled, washed twice in distilled water, and incubated for 30 min in a rotating 37° water bath with guinea pig sera using 25 mg of zymosan per ml of serum. After the zymosan had been removed by centrifugation, the supernatant was incubated at 56° for 30 min. The heated serum was stored in small aliquots at −70° and a sample was analyzed

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by gel filtration on a Sephadex G-75 column (50 × 1.5 cm) equilibrated with Tyrode's solution.

Cell preparation. Cells obtained by peritoneal lavage of guinea pigs with normal sterile saline (9) were centrifuged for 5 min at 100*g*, washed in Tyrode's solution, and resuspended in T-HSA at a concentration of 10^7 cells/mm^3. The final preparation contained approximately 30% eosinophils, the remaining cells being mononuclear in type.

Chemotactic assay. Chemotaxis was tested using a modified Boyden chamber (10), especially adapted for analysis of small volumes (Neuroprobe Company, Bethesda, Md.). The chemotactants, in a volume of 0.030 ml, were placed in the bottom chamber, and the cell suspension was placed on the top. The chambers were separated by an 8-μm Millipore membrane (Millipore Corporation, Bedford, Mass.) (11). For study of the capacity of drugs to alter chemotaxis, 0.05 ml of a solution of drug and 0.05 ml of the cell suspension were added to the top chamber. After the chambers had been incubated for 3 hr at 37° the filters were removed and stained with Carrazi's hematoxylin and Chromotrope 2R (11). Migration of the eosinophils was measured using the "in filter count" method (12). In this method an arbitrary point 90 μm from the top of the stained membrane was chosen and counts were made there and at every 5 μm until the bottom of the filter was reached. All the eosinophils with distinct nuclei were counted at each level. The chemotactic counts were expressed as the mean cell count of five high-power fields (11). The chambers were processed in duplicate. In some experiments the test drug was placed in the lower compartment with the same volume of chemotactant, in order to determine whether the action of the drug was directly on the cells or on the chemotactant. Because of reports of cells dropping off the top of the filter and interfering with the final chemotactic count (13), the liquid remaining in the lower compartment after the 3-hr incubation period was pelleted on a slide using a cytocentrifuge and was stained with Wright's stain. No cells were found.

Viability assay. The effects of hydrocortisone sodium succinate, methylprednisolone sodium succinate, and chloroquine hy chloride on the viability of the eosino were investigated by trypan blue exlu After the cells were incubated at 37° f hr with the different concentrations o drugs, the cells were diluted in 0.5% tr blue in saline and examined microscopi after 10 min. The number of stained per 100 cells was taken as the numb killed cells. The results were compared a control consisting of cells in T-HSA.

Results. Gel filtration of the chem tants. As shown in Fig. 1, gel filtratio the lung tissue extract on the Sephade 25 column showed chemotactic activi fractions eluting at volumes roughly c sponding to glycyl-L-tyrosine, indic that the chemotactic substance had a m ular weight approximately the same as of ECF-A (mol wt 360-390) (14). Frac obtained by gel filtration of zym treated guinea pig sera were tested chemotactic activity as shown in Fig. 2. activity emerged from the column sli before cytochrome *C* (mol wt 12,000) well before bacitracin (mol wt 1400), cating that the molecular weight of chemotactant is approximately the sam that of guinea pig C5a (mol wt 15,000)

Effect of corticosteroids on chemot Corticosteroids produced a dose-relate hibition of chemotaxis induced by ECF-A and C5a with concentrations r ing from 0.15 to 2 mg/ml (examples sh in Figs. 3 and 4) corresponding to 1. 10^{-4} to 4.1×10^{-3} *M*, respectively. M ylprednisolone appeared to be more in ting than hydrocortisone. In five a marked variation in response was n

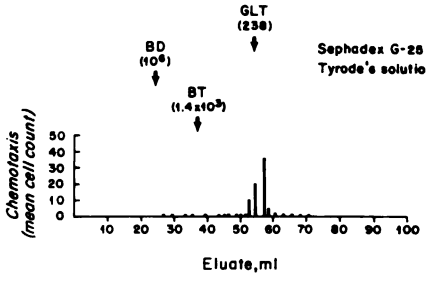
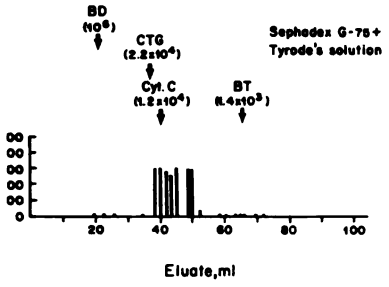
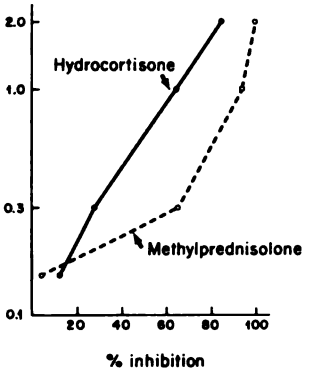


FIG. 1. Analysis of ECF-A chemotactic activ gel filtration of the lung extract. Markers are dextran (BD), bacitracin (BT), and glycyl-L-ty (GLT).



. Analysis of C5a chemotactic activity by gel of the zymosan-treated guinea pig serum. are blue dextran (BD), chymotrypsinogen ytochrome *C* (Cyt. C), and bacitracin (BT).



. Inhibition of ECF-A-mediated eosinophil by hydrocortisone sodium succinate and dnisolone sodium succinate.

ls from different guinea pigs, but a ated inhibition was always present. hydrocortisone, in concentrations from 0.1 to 2 mg/ml, was mixed F-A in the lower compartment, no n of the eosinophil migration was lso, hydrocortisone together with concentrations up to 0.5 mg/ml o inhibition of chemotaxis. Concen- of 1 and 2 mg/ml in the chemotac- partment caused 13% inhibition of hil migration, whereas 65 and 80% n, respectively, were caused by the ses when placed in the cell compart- hese results suggest that the corti- d action is directly on the cell and he chemotactant. In order to deter- hether inhibitory action was re- to glucocorticosteroids, three other , estrone, estradiol, and testoster- ere analyzed for their eosinophil ictic inhibitory activity using ECF-A as the chemotactant. As a control, the diluent alone, 0.5% ethanol, was employed. In the studies by Ward (5) this concentration of ethanol was not inhibitory for the chemotaxis of polymorphonuclear neutrophils *in vitro*. However, in two assays performed with eosinophils, 20 and 40% inhibition was observed when 0.5% ethanol was mixed with cells as compared to the control without ethanol. When comparing the results for steroids to the 0.5% ethanol control, there was either no difference or the steroids were only slightly more inhibitory (approximately 10%).

Effect of chloroquine hydrochloride on chemotaxis. Chloroquine hydrochloride was tested for inhibition of eosinophil chemotaxis in doses ranging from 10^{-6} to 10^{-1} *M*. As shown in Fig. 5 an inhibitory effect was observed with lower concentrations than in the case of the corticosteroids. When placed in the bottom chamber with C5a, chloroquine, in doses ranging from 10^{-6} to 10^{-4} *M*, caused no inhibition and, at a concentra-

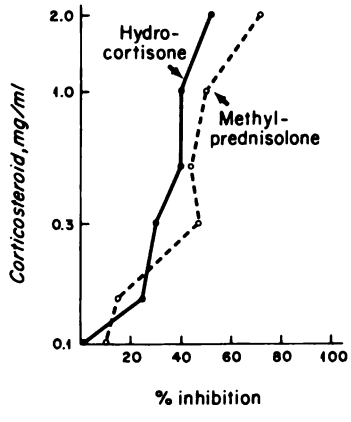


FIG. 4. Inhibition of C5a-mediated eosinophil migration by hydrocortisone and methylprednisolone.

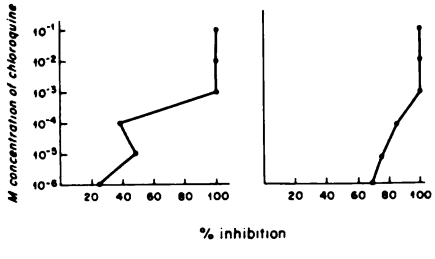


FIG. 5. Inhibition of ECF-A and C5a activity by chloroquine.

tion of 10^{-3} *M*, caused 15% inhibition compared to 100% when placed with the cells, thus suggesting a direct action of this drug on the eosinophils.

Viability assays. The mechanism of action of the corticosteroids on the cells remains to be explained. That they do not kill the eosinophils was demonstrated by the trypan blue exclusion test. In two assays the number of killed cells was much lower than the control (Table I), suggesting that the effects of the drugs were to improve the viability of the eosinophils. When eosinophils were incubated with chloroquine in the trypan blue exclusion test, slightly fewer dead cells than in the control were obtained at chloroquine concentrations up to 10^{-4} *M*. Above this concentration, trypan blue precipitated and no conclusions could be drawn.

Discussion. The results obtained in this study indicate that two corticosteroids, hydrocortisone sodium succinate and methylprednisolone sodium succinate, and chloroquine hydrochloride cause a drug-related inhibition of eosinophil migration *in vitro*, and these results are in agreement with the results of Goetzl and his associates who

TABLE I. EOSINOPHIL VIABILITY

	Staining by trypan blue (%)	
	Expt 1	Expt 2
Eosinophils in Tyrode's 2% human serum albumin	15	24
Hydrocortisone		
0.2 mg/ml	0	–[a]
1 mg/ml	1	12
2 mg/ml	4	7
4 mg/ml	2	4
8 mg/ml	4	3
Methylprednisolone		
0.2 mg/ml	5	–[a]
1 mg/ml	3	1
2 mg/ml	5	0
4 mg/ml	10	4
Chloroquine hydrochloride		
10^{-6} *M*		30
10^{-5} *M*		18
10^{-4} *M*		26
10^{-3} *M*		–[b]
10^{-2} *M*		–[b]

[a] Not determined.
[b] Trypan blue precipitated.

reported that hydrocortisone and hy chloroquine suppress directed migra eosinophils (15). In both studies th of corticosteroids necessary to obt effect were much above physiologica concentrations of hydrocortisone an above high pharmacological blood For example, intravenous administra 100 mg of hydrocortisone yields levels of up to 0.0018 mg/ml (16). treatment of patients with asthma of hydrocortisone might be administ a single intravenous injection, follo a continuous intravenous infusion mg and finally another intravenous in of 200 mg a few hours later. In this si the plasma levels of hydrocortisone be very high, conceivably as high mg/ml. However, as shown in Fig. levels would be 20-fold lower tha used in our experiments; our lowe was about 0.2 mg/ml. We do no whether corticosteroids or their der might be concentrated in cells to them more potent physiologically. rect action of these drugs on the ce confirmed by placing them with the tactant in the lower compartment concentrations caused minimal inl compared with much more pronoun hibition seen when drugs were pla rectly with the cell suspensions. The r inhibition seen with the drugs in the otactant compartment may be expla drug diffusion into the upper compa Cytotoxic experiments demonstrate neither corticosteroids nor chlo killed the eosinophils. The mechan actions of these drugs are unclear. A chloroquine affects the morphology o ophil granules in man and the r 18), there is no evidence that this alters the ability of the cell to show d migration. Lysosomal membrane st tion could be important (8, 19, 20). chemotaxis of neutrophils serine es are activated (21), but these have n studied in relation to eosinophilota have the effects of corticosteroids or esterases been reported.

Summary. In an *in vitro* chemotact using a modified Boyden chamber, tion of anti-inflammatory agents on

vas studied. Hydrocortisone so-
ıate, methylprednisolone sodium
and chloroquine hydrochloride
dose-related inhibition of migra-
inophils to ECF-A and C5a. The
action appeared to be directly on
nd it was not caused by lysis of

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Effects of Thyrotropin-Releasing Hormone on Plasma Thyroxine in the Calf: Comparison of Intraventricular and Intravenous Injection Routes[1] (40007)

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Several investigators have examined the possible role of cerebrospinal fluid (CSF) in the transport of thyrotropin-releasing hormone (TRH) to the pituitary gland by comparing the relative effectiveness of intraventricular and intravascular TRH in altering pituitary or thyroid function. On the basis that CSF offers a smaller dilution volume and a longer half-life for TRH (1), it was reasoned that, if CSF is involved in TRH transfer, intraventricular TRH injection might be more effective in stimulating the pituitary gland than intravenous injection. However, Gordon *et al.* (2) found that intraventricular TRH was much less effective in elevating plasma thyrotropin (TSH) than jugular injection, while Oliver *et al.* (3) reported that the two routes were about equal. Kendall *et al.* (1) concluded that lateral ventricular and jugular injections of TRH were equal in stimulating iodine release from the thyroid. These observations indicate that, although intraventricularly injected TRH does reach the pituitary gland, TRH by this route is not more effective than by the intravenous route. This suggests that CSF may not be important for the transport of TRH.

Each of these studies utilized rats anesthetized with agents known to alter the concentrations of plasma hormones including TSH (4-7). Thus, important differences between the two routes of TRH administration may have been masked in these studies by the anesthetic agents. The present study compares the relative effectiveness of various doses of intraventricular and intravascular TRH to alter thyroid gland function in unanesthetized calves in order to examine the possibility of CSF transport of TRH.

Materials and methods. The Guernsey bull calves (40-60 kg, 4-6 months old) used in these studies were housed in a controlled environment at 20 ± 1° and with 14 hr of light per day. A maintenance ration was fed twice daily and water was provided *ad libitum*. The calves were prepared with lateral ventricular guide cannulae at least 1 month before the start of the experiments (8). The patency of the ventricular cannula was confirmed before and after each study by removing clear CSF. Cannula locations were also confirmed by dissection at autopsy.

In the first study, three calves were treated with intraventricular and intravascular injection of synthetic TRH (Abbott Laboratories, North Chicago) in 0.9% saline at doses of 0, 12.5, 25, 50, 100, and 200 μg. A minimum of 2 days lapsed between the start of these individual treatments. After a 200-μg dose, however, the next treatment was not given until 4 days later. Examination of the data indicated that these intervals were sufficient to allow for return of thyroxine to basal levels. All injection solutions were sterilized by filtration. Chronic jugular vein catheters, in place several days before the start of the experiment, were used for blood sampling and injection. Injection volumes were 0.1 ml for the ventricular route and 1 ml for the jugular route. For lateral ventricular injection, the injector cannula, tubing, and syringe were completely prefilled with the treatment solution. After ventricular injection, the cannula was left in place for at least 1 min. Following jugular injection of the treatment solution, the catheter was flushed with 3 ml of saline. Six milliliters of blood were withdrawn from the jugular vein with heparinized syringes 10 min before injection, and then 10, 20, and 30 min and 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, and 23 hr after

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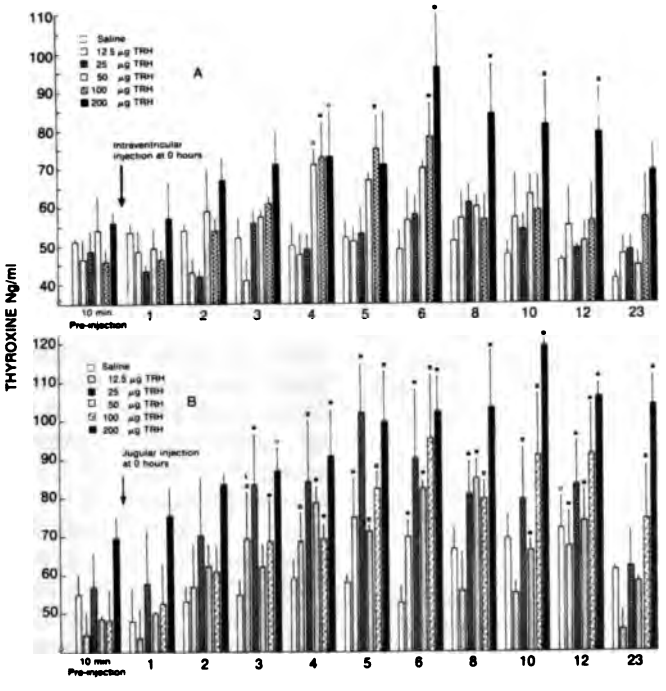
ventricular or jugular injection. After ugation, the plasma was stored frozen ssay.

he second study, five calves (three Study I) were treated by the two with saline and 100 μg of TRH. for 8- and 10-hr samples, blood was awn at the times listed for Study I. ther procedures used were as de- l above.

roxine was measured in duplicate 25- sma samples using the radioimmu- y method of Premachandra and Ibra-) as modified by Vanjonack (10), ng the use of the charcoal separation ique of Herbert *et al.* (11). The coeffi- of variation for pooled plasma sam- ere 9.1% between assays of 11.1% assays. Data were analyzed by anal- variance; individual sample means compared at the 0.05 level by the gnificant difference procedure in Study I and by Duncan's new multiple-range test in Study II (12). The linear regression between thyroxine levels and first 11 sampling times was also calculated for each treatment group (13). All TRH injection groups, except the 12.5-μg intraventricular group, showed significant ($P < 0.0001$) linear regressions. Goodness of fit tests on these regressions indicated that the linear equations were an acceptable representation of the data.

Results. In the dose-response study (I), thyroxine levels reached their maxima 4 to 8 hr after intraventricular injection of TRH (Fig. 1A). Only in the 200-μg dose group did thyroxine levels remain significantly elevated 8, 10, and 12 hr after treatment. Twenty-three hours after injection, none of the groups showed significantly elevated thyroxine levels. On the basis of comparing means, there was no indication that the 12.5- and 25-μg dose groups were effective



1. Plasma thyroxine concentrations at various times following intraventricular (A) and jugular (B) i of saline and five dose levels of thyrotropin-releasing hormone (TRH). Each bar represents the mean cate determinations from three calves. Standard error of the mean is shown as a vertical line. A nt difference between a postinjection mean and a corresponding preinjection mean is shown either as a < 0.05) or an open circle ($P < 0.06$). Postinjection means for the 10-, 20-, 30-, and 90-min samples rn on Fig. 2.

at any time. There was also no indication that thyroxine levels in any group at 10, 20, 30, or 90 min after injection were significantly changed from preinjection levels (these means are plotted on Fig. 2A).

Using only the first 6 hr of intraventricular data, regression analysis indicated that thyroxine concentrations increased linearly with time in the 200-, 100-, 50-, and 25-μg dosage groups (Fig. 2A). Slopes of the regression lines for these groups were different from zero ($P < 0.0001$). With saline and 12.5-μg TRH treatments, no significant linear regression was found. This analysis suggests that intraventricular TRH at dosages of 25 μg or more are effective in elevating plasma thyroxine levels.

Following jugular injection of TRH (Study I), thyroxine concentrations increased significantly ($P < 0.05$) from preinjection level to peak at 10 hr with 200 μg of TRH, at 6 hr with 100 μg, at 8 hr with 50 μg, at 5 hr with 25 μg (N.S.), and at 5 hr with 12.5 μg (Fig. 1B). At the 23-hr sample, only the 100- and 200-μg groups showed significantly elevated thyroxine levels ($P < 0.05$). The earliest significant ($P < 0.05$) elevations were seen at 3 hr when levels were increased above the preinjection levels in all groups except the 50-μg TRH.

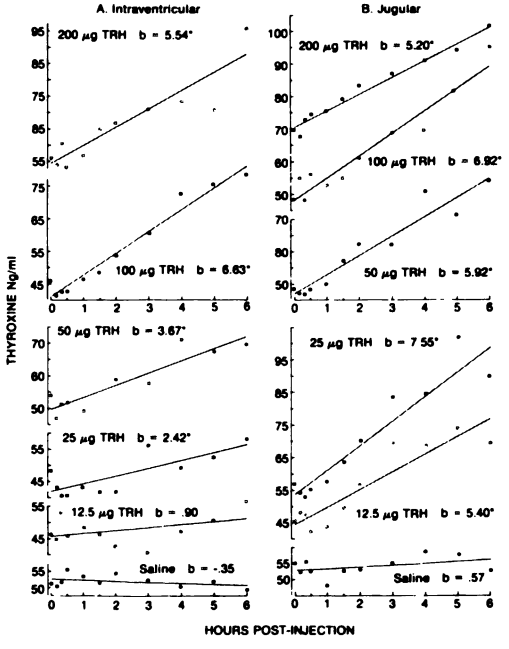
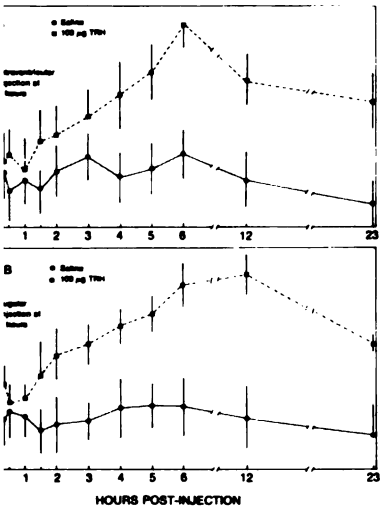

A. Intraventricular
B. Jugular
200 µg TRH b = 5.54*
100 µg TRH b = 6.63*
50 µg TRH b = 3.67*
25 µg TRH b = 2.42*
12.5 µg TRH b = .90
Saline b = -.35
200 µg TRH b = 5.20*
100 µg TRH b = 6.92*
50 µg TRH b = 5.92*
25 µg TRH b = 7.55*
12.5 µg TRH b = 5.40*
Saline b = .57
THYROXINE Ng/ml
HOURS POST-INJECTION

FIG. 2. Plasma thyroxine concentrations for the first 6 hr after intraventricular (A) and jugular (B) injection of saline and five dose levels of thyrotropin-releasing hormone (TRH). Each of the data points is the mean of determinations from three calves. Solid lines were fitted to individual values by the least-squares method. Asterisks show slopes that are different from zero ($P < 0.0001$).

Regression analysis of the first 6 hr of jugular data indicated that plasma thyroxine concentrations increased linearly with time with all TRH dosage groups (Fig. 2B). Slopes of all the TRH linear regression lines were different from zero ($P < 0.0001$) but none were different from each other, while saline injection data did not yield a significant linear regression. A t test comparison of regression slopes for the same doses between the two routes of TRH administration indicated significant differences ($P < 0.05$) between the routes with the 12.5-, 25-, and 50-μg dosage groups, but no differences with the 100- and 200-μg.

In the second study (II), involving five calves, comparisons were made between routes after injection of saline and 100 μg of TRH. By comparison to preinjection levels, thyroxine was elevated in both TRH groups at all sampling times from 1.5 to 23 hr (Fig. 3). Regression analysis indicated that thyroxine concentrations in both TRH-injected groups varied linearly with time for the first 6 hr. The slopes of the lines were 7.10 for the intraventricular route and 6.18 for the jugular route. These slopes were not significantly different from each other or from the slopes of 100-μg dose groups in Study I; however, they were different from zero ($P < 0.0001$). Lines fitted by least-squares method for the saline groups had slopes of 1.78 for the intraventricular route and .75 for the jugular routes; neither was significantly different from zero.

Discussion. The thyroxine response to TRH appears to be directly related to the TRH dosage. This relationship is clearest with ventricular injection at the 6-hr sample time (Fig. 1A) and with the rank order of change of thyroxine concentrations from preinjection to peak levels. Except for the 25-μg dose group, equivalent data from the jugular route treatment also shows a dose-response relationship (see Fig. 1B). Comparing the two routes, Kendall *et al.* (1) noted that thyroidal iodine release varied



Plasma thyroxine concentrations at various intraventricular (A) or jugular (B) injection ıd 100 μg of thyrotropin-releasing hormone udy II). Each point shows the mean of five the standard error.

vith the TRH dose; while Oliver *et* und dose-related changes in plasma centration.

highest dose levels (100 μg ≡ 2 00 μg ≡ 4 μg/kg), the effectiveness. to elevate plasma thyroxine was nilar for the intraventricular and outes (compare Figs. 1, 2, and 3). *et al.* (1), using etherized rats and μg of TRH/kg, also found that the TRH to stimulate thyroidal iodine vas equal for the two routes. Simi- iver *et al.* (3) observed little differ- ween the two routes in terms of the ness of 300 ng of TRH/kg to ele- ma TSH levels in rats anesthetized ıtobarbital. These observations in- ıat intraventricular TRH is trans- ut of the brain and is effective in ng the pituitary-thyroid axis. The / of responses with anesthetized nesthetized animals suggests that, high TRH dose levels, anesthesia alter the ability of intraventricular stimulate the pituitary-thyroid axis. : lower dose levels, the ability of elevate thyroxine concentrations ırly dependent upon the route of ration. All TRH dose levels by the route were effective in elevating thyroxine concentrations, whereas only the three highest doses were clearly effective by the intraventricular route. Intraventricular 12.5-μg TRH (≡250 ng/kg) was not effective and the potency of 25 μg (≡500 ng/kg) was only marginal. Oliver *et al.* (3) reported that with anesthetized rats, 30 ng of TRH/kg injected into the cerebral ventricles produced a much smaller increase in plasma TSH levels than when the same dose was given by jugular injection. Using pentobarbital-anesthetized rats, Gordon *et al.* (2) injected 50 ng of TRH (≡170 ng/kg) at various sites and found that intrapituitary injection resulted in the greatest increase in plasma TSH while the intraventricular route produced the smallest increase. These observations indicate that when lower doses of TRH are used, ventricular TRH injection is clearly less effective than intravascular injection. Since the results from anesthetized animals are similar to those seen with the unanesthetized calves, anesthesia is apparently not a major factor in impeding the action of these lower doses of intraventricular TRH.

It is not possible, at the present time, to evaluate critically the relative importance of CSF transport of TRH because we do not know what portion, if any, of the total hypophysiotropic TRH actually travels through CSF to reach the pituitary gland. What the present study offers are answers to several questions important to the transport issue. Our results are consistent with previous reports (see above) which show that TRH can transfer from CSF to blood to stimulate pituitary and thyroid secretion. This transfer process is apparently not affected by anesthetics known to influence TSH secretion. The effectiveness of TRH to stimulate the pituitary-thyroid axis is clearly not identical for the two routes of administration. If the two routes were equal, this would suggest that movement of TRH from CSF to the pituitary gland is dependent primarily upon the same physical forces governing distribution in the circulatory system. Since the transfer of ventricular TRH to the pituitary gland appears to be impeded by comparison to vascular injection, cellular activity might be involved at some point in the transfer of TRH from CSF to blood.

Several observations suggest that the median eminence is involved in the transfer of substances from CSF to portal capillaries. Goldgefter (14) found that tritiated LH-RH injected into the lateral ventricle penetrated into subependymal areas of the median eminence by ordinary diffusion and accumulated in cells in the external zone by a nondiffusion process. Ependymal cells of the median eminence have been demonstrated to take up labeled LH-RH (15) and TRH (16, 17) following intraventricular injection. Labeled TRH is also detected in the anterior pituitary gland, as well as the median eminence, shortly after third ventricle infusion (18). Oliver *et al.* (3) found that after lateral ventricle injection of labeled TRH, both the appearance of TRH in portal blood and the increase in plasma TSH were delayed by comparison to intravenous TRH injection. Although our results do not indicate a route or mechanism for TRH action, the above studies suggest that transport of TRH by the median eminence may impede TRH transfer from CSF to blood and thus could account for the reduced effectiveness of the intraventricular route.

Summary. Various doses of TRH (12.5, 25, 50, 100, and 200 μg) were injected into the lateral ventricle and jugular vein of unanesthetized calves to examine the possibility of CSF transport of thyrotropin-releasing hormone (TRH). At higher doses (100 and 200 μg), there was no major difference in effectiveness of jugular and ventricular TRH to elevate thyroxine levels. Thyroxine levels were significantly elevated as early as 1.5 hr and peaks occurred 6 to 12 hr after 100 μg of TRH. At the lower doses (<100 μg), the effectiveness of TRH to elevate thyroxine levels was clearly route dependent. All TRH doses (<100 μg) by jugular injection were effective, while only the 50-μg intraventricular dose was clearly effective. These observations indirectly indicate that TRH is transferred from CSF to the vascular system. This transfer process, possibly involving cells of the median eminence, may impede the movement of TRH to blood and thus account for the reduced effectiveness of intraventricular TRH.

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Sulfur Amino Acid Activity of D- and L-Homocysteine for Chicks[1] (40008)

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ıong the indispensable amino acids, ionine is unique because both of its ers have equal nutritive value when d to a diet at levels near the animal's ry requirement (1). This raises spec- ın concerning the biopotency of the D L isomers of homocysteine, since ho- steine is a normal intermediate in conversion of methionine to cysteine. ıitive information is lacking concern- fficacy of the isomers of homocysteine urces of methionine per se or cysteine e.

virtually all nutritional research with ɔcysteine, the DL-racemic mixture has used. Moreover, cysteine status of ınimal has not been clearly defined. resulting data have therefore been ult to interpret. Conflicting reports ar in the literature (2-9) regarding fficacy of homocysteine as a source of r amino acids (SAA). The earliest by du Vigneaud *et al.* (2) indicated ılation of growth in rats when DL- ɔcysteine was added to a cystine-defi- diet. They suggested, however, that isomers were not equal in biopotency. ubsequent work, Dyer and du Vig- d (4) observed that growth of rats fed ıocysteine in a cystine-deficient diet equal to that of rats fed the L isomer, ugh both isomers were inferior to L- ine.

e transmethylation of homocysteine ethionine (Fig. 1) has become a gen- / accepted concept based upon early th data with rats and chicks (5-7). original research was done without ıid of a carefully defined purified diet as such, nutrient imbalances were non. Rose and Rice (5) observed re- ses in rats to DL-homocysteine when d to a SAA-free diet, but growth was still inferior to that of rats fed DL-methionine. du Vigneaud and co-workers (6), feeding a purified diet similar to that of Rose and Rice, observed that growth of rats fed DL-homocysteine equaled that of rats fed DL-methionine. It should be noted, however, that rats fed DL-methionine gained only 1 g/day, a growth rate of questionable magnitude for evaluating transmethylation activity.

The purpose of our assays was to evaluate the D and L isomers of homocysteine[3] as sources of either methionine or cysteine for chicks fed a purified L-amino acid diet that allowed strict control of methionine and cysteine status.

Materials and methods. Male chicks resulting from the cross of New Hampshire males and Columbian females were used in all assays. Care of chicks prior to the start of each assay and procedures of experimental allotment have been described (10). The basal diet (Table I) was devoid of sulfur amino acids, and all additions were made at the expense of cornstarch. Feed and water were offered *ad libitum*. Growth and feed efficiency were measured. Data were analyzed by analysis of variance techniques, and pooled standard errors were calculated for each response parameter.

Assay 1 was designed to determine the efficacy of the D and L isomers of homocysteine as sources of methionine or cysteine. A cystine-deficient diet series and a methionine-deficient diet series were fortified with either D- or L-homocysteine, and performance was compared with an equimo-

ıtroduced by Willard J. Visek.

ɔ whom correspondence should be addressed.

[3] L- and D-homocysteine were provided as homocysteinethiolactone hydrochloride (De Gussa, Inc.), a compound equal to homocysteine in biological activity. Male chicks in our laboratory fed SAA-deficient diets to which were added isosulfurous quantities of DL-homocysteine or DL-homocysteine-thiolactone·HCl from Day 8 to Day 20 posthatching gained 129.5 and 129.6 g, respectively.

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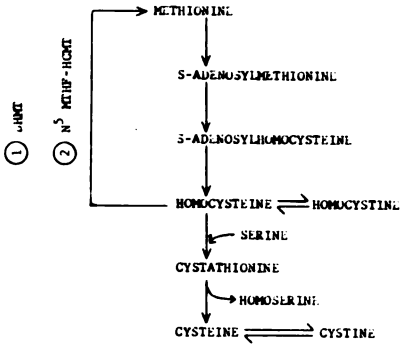

FIG. 1. Scheme of homocysteine interconversion. ① BHMT, Betaine-homocysteine methyltransferase; ② N^5 MTHF-HCMT, N^5-methyltetrahydrofolate-homocysteine methyltransferase.

lar level of L-cystine or L-methionine, respectively. In the cysteine series, sufficient methionine (0.30%) was present to meet the chick's physiological requirement and, in the methionine series, sufficient cystine (0.25%) was provided to cover the physiological requirement for cystine (11).

Assay 2 was designed to ascertain if performance of chicks fed graded levels of L-homocysteine as a source of methionine could be made equal to that of chicks fed L-methionine itself. L-Cystine was included in all diets at 0.35% to ensure that it would not be growth limiting. Treatments (Table III) consisted of graded levels of L-methionine to establish a standard curve and graded levels of L-homocysteine to generate a dose-response curve that could be related to the L-methionine standard curve. The slope-ratio approach (10, 11) was used to determine the efficacy of L-homocysteine as a source of methionine.

Comparative growth of chicks fed L-homocysteine as a source of methionine in diets containing two levels of choline was evaluated in assay 3. All diets were made adequate in L-cystine, and isosulfurous levels of L-methionine and L-homocysteine were fed. Choline chloride was included in the diet at either 0.20 or 0.50%.

Assay 4 evaluated homocysteine in diets with and without dietary cystine. Work by various researchers (2-9), which had shown homocysteine to be a good source of methionine, was done with diets that were limiting in both cysteine and methionine. Thus, homocysteine was serving source of total sulfur amino acids (T in these studies rather than as a sou methionine alone. Assay 4 was the designed to determine if methionin cysteine must both be limiting in for homocysteine to be utilized effic DL-Homocysteine[4] was used in this due to exhaustion of the supply of L- cysteinethiolactone hydrochloride.

Results and discussion. Chicks homocysteine as a source of cy gained as fast and as efficiently as fed L-cystine (Table II). Relative gai reduced to 68%, however, for chicks homocysteine. Both L- and D-homocy were poorly utilized as sources of m nine in diets where the cysteine re ment had been met by provision of L-cystine. Relative gain for the 8-day for chicks fed L-homocysteine was that of chicks fed L-methionine, an 7% when D-homocysteine was fed. results indicate that the chick fed tine-adequate diet cannot efficiently vert either L- or D-homocysteine to onine. In contrast, chicks fed methi adequate diets can convert homocy to cysteine much more efficiently. T knowledge, this has been the first w quantify the transmethylation conv of D- and L-homocysteine to meth under conditions of cysteine ade However, homocysteine utilizatior been studied by Dyer and du Vignea and du Vigneaud *et al.* (2) in ra diets deficient in cystine.

Results of assay 2 are shown in III. Chicks responded linearly to levels of L-methionine resulting in a dard curve with a slope of 36.44 g o mmole of L-methionine intake as in Fig. 2. Chicks fed graded levels homocysteine responded in a manne described by a broken-line least-s model as shown in Fig. 2. Chick intakes of L-homocysteine up to mmole gained 28.30 g/mmole of Chicks with intakes greater than mmole of L-homocysteine gained onl

[4] DL-Homocysteine supplied by De Guss West Germany.

TABLE I. COMPOSITION OF BASAL DIET

Ingredient	(%)	Amino acid mixture	(g/19.78 g)
ornstarch	to 100	L-Arginine·HCl	1.15
mino acid mixture	19.78	L-Histidine·HCl·H_2O	0.45
orn oil	15.00	L-Lysine·HCl	1.14
ılt mixture[a]	5.37	L-Tyrosine	0.45
ellulose[b]	3.00	L-Tryptophan	0.15
aHCO$_3$	1.00	L-Phenylalanine	0.50
holine chloride	0.20	L-Methionine	–
itamins[a]	0.20	L-Cystine	–
-Tocopherol acetate (20 mg/kg)	+	L-Threonine	0.65
thoxyquin (125 mg/kg)	+	L-Leucine	1.00
		L-Isoleucine	0.60
		L-Valine	0.69
		Glycine	0.60
		L-Proline	0.40
		L-Glutamic acid	12.00
		Total	19.78

[a] Katz and Baker (10).
[b] Solka Floc, Brown Company, Chicago, Ill.

TABLE II. PERFORMANCE OF CHICKS FED D- AND L-HOMOCYSTEINE AS A SOURCE OF EITHER METHIONINE OR CYSTINE (ASSAY 1)[a]

Diet	Level of SAA (%)[b]				Gain (g)	Relative gain (%)	Gain/feed
	L-M	L-C	L-HC	D-HC			
1	0.30	–	–	–	39.4	35	0.40
2	0.30	0.25	–	–	113.9	100	0.72
3	0.30	–	0.28[c]	–	110.0	97	0.71
4	0.30	–	–	0.28[c]	77.4	68	0.59
5	–	0.25	0.27[d]	–	32.3	28	0.44
6	–	0.25	–	0.27[d]	7.9	7	0.16
7	0.30	0.25	0.28	–	103.8	91	0.71
8	0.30	0.25	–	0.28	101.2	89	0.72
ıoled SE					2.4	3	0.01

[a] Average of triplicate groups of seven male chicks for the period 8 to 16 days posthatching; average initial ;ight was 82 g.
[b] SAA, Sulfur amino acids; L-M, L-methionine; L-C, L-cystine; D and L isomers of homocysteine (HC) were ɔvided as homocysteinethiolactone hydrochloride.
[c] Isosulfurous to 0.25% L-cystine.
[d] Isosulfurous to 0.30% L-methionine.

mmole. Using the slope-ratio technique, vels of intake up to 0.765 mmole resulted 0.777 mmole of L-methionine activity/ mole of L-homocysteine (i.e., 28.30 g of ıin/mmole of L-homocysteine ÷ 36.44 g of ıin/mmole of L-methionine). Therefore, homocysteine, at intakes less than 0.765 mole, was 77.7% as efficacious as L-me- ıionine in supporting growth. Similarly, hen intake exceeded 0.765 mmole the ficiency was reduced to 15.4% on a molar ısis. This type of response is similar to ıat seen in nutrient requirement studies here no additional growth response oc- ırs when intake of a nutrient is in excess its physiological requirement for growth. In this case, however, the response plateau would indicate a saturation of the transmethylation activity for reasons that can only be speculated. The two-slope response seen here may be the reason for conflicting data in the literature. Careful examination of the amount of homocysteine supplementation relative to this response curve is therefore important when evaluating the degree of homocysteine transmethylation.

Amos and co-workers (9) recently reported weight gains of 80 to 90% for rats fed homocysteine (provided as DL-homocysteinethiolactone·HCl) relative to rats fed DL-methionine. However, the basal

TABLE III. PERFORMANCE OF CHICKS FED GRADED LEVELS OF L-HOMOCYSTEINE AS A SOURCE OF METHIONINE (ASSAY 2)[a,b]

Diet[c]	Source of SAA (%)[d] L-M	L-HC	Gain (g)	Gain/feed
1	–	–	−3.5	−0.50
2	0.10	–	5.8	0.16
3	0.20	–	38.9	0.55
4	0.30	–	72.5	0.69
5	–	0.103	3.2	0.10
6	–	0.206	8.9	0.28
7	–	0.309	19.1	0.42
8	–	0.515	24.7	0.46
9	–	0.721	27.3	0.54
Pooled SE			2.3	0.04

[a] Average of triplicate groups of five male chicks for the period 8 to 14 days posthatching; average initial weight was 64 g.

[b] Diets 2 and 5, 3 and 6, and 4 and 7 are isosulfurous.

[c] All diets contained 0.35% L-cystine.

[d] L-Homocysteine (L-HC) was provided as L-homocysteinethiolactone hydrochloride; L-M, L-methionine.

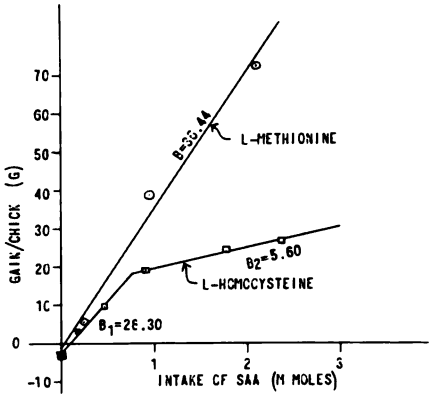

FIG. 2. Standard curve of gain per chick as a function of L-methionine intake and the broken-line response curve of L-homocysteine as a function of intake. B, B_1, and B_2 indicate slope values based on regression analysis of weight gain as a function of dietary intake.

diet of isolated soybean protein contained 0.2% methionine. Their 80-90% efficacy results were based on total gain of rats fed the basal diet supplemented with DL-homocysteine and DL-methionine. This approach actually compares the TSAA content of each diet and not the the gain due to DL-homocysteine per se. When weight gain due to homocysteine per se is plotted against actual intake, a value of 35% utilization of homocysteine as a source of TSAA is the result. This is in agreement with our results showing that homocysteine is a poor source of methionine.

The plateau in growth at higher levels of homocysteine as seen in Fig. 2 may be due to a depletion of methyl donors for the transmethylation reaction. Klose and Almquist (7) reported poor gains in chicks fed DL-homocysteine in an arachin-based diet containing 0.20% supplemental choline chloride relative to those of chicks fed DL-methionine. When choline chloride was increased to 0.50%, weight gains were equal for both sources of SAA. The significance of this observation is hard to determine, however, because growth of the chicks was very poor. Chicks fed their basal diet which contained close to 0.5% SAA lost weight. Thus, some nutrient other than SAA must have been growth limiting.

Our basal diet contained 0.2% choline chloride, which is almost three times the growth requirement for chicks of this strain under our experimental conditions (12). It is therefore unlikely that choline was limiting in these assays. Results of assay 3 (Table IV) indicate that chicks fed L-homocysteine in the presence of 0.5% choline chloride showed no improvement in weight gain over chicks fed 0.2% choline chloride. Weight gains of chicks fed L-homocysteine at either level of choline chloride were inferior ($P < 0.01$) to those fed L-methionine.

TABLE IV. EFFECT OF EXCESS DIETARY CHOLINE ON THE EFFICACY OF L-HOMOCYSTEINE AS A SOURCE OF METHIONINE FOR THE YOUNG CHICK (ASSAY 3)[a]

Diet[b]	Level of SAA (%)[c] L-M	L-HC	Choline-Cl (%)	Gain (g)	Gain/feed
1	0.314	–	0.20	81.5	0.68
2	–	0.323	0.20	32.5	0.47
3	–	0.323	0.50	32.7	0.43
Pooled SE				2.8	0.03

[a] Average of triplicate groups of five male chicks for the period 16 to 21 days posthatching; average initial weight was 99 g.

[b] All diets contained 0.35% L-cystine.

[c] L-M, L-methionine; L-HC, L-homocysteine provided as L-homocysteinethiolactone hydrochloride; all additions are isosulfurous.

BLE V. PERFORMANCE OF CHICKS FED DL-MOCYSTEINE AS A SOURCE OF TOTAL SULFUR AMINO ACIDS (ASSAY 4)[a]

Level of SAA (%)[b]				
L-M	L-C	DL-HC	Gain (g)	Gain/feed
0.50	–	–	114.2	0.63
–	–	0.45	53.9	0.49
–	–	0.90	61.2	0.60
0.25	0.25	–	114.5	0.61
–	0.25	0.45	60.4	0.57
–	0.25	0.91	54.6	0.57
SE			4.4	0.02

'erage of triplicate groups of seven male chicks period 7 to 17 days posthatching; average 'eight was 66 g.
M, L-methionine; L-C, L-cystine; DL-HC, DL-'steine.

ılts of assay 4 (Table V) indicate chicks fed DL-homocysteine as a of SAA perform similarly to chicks -homocysteine as a source of methi- per se. Since it was established in 1 that homocysteine has a high ıe-sparing value, the utilization of ysteine as a source of SAA appears limited by its methionine activity. esults in these assays clearly indicate ıe chick fed a crystalline amino acid ınnot efficiently convert either D- or ocysteine to methionine via trans- lation. It would thus appear that (a) some factor necessary to stimu- ransmethylation activity is missing rotein-free, L-amino acid diet or (b) species, in fact, cannot under any ıstances meet their entire require- for dietary methionine via methyla- f homocysteine.

ımary. Young male chicks were fed lline L-amino acid diets in assays ıed to evaluate the capacity of D- -homocysteine to supply methionine ysteine. L-Homocysteine had a cys- ɔaring value of 100% and a methio- paring value of 28% at the level fed. nocysteine was utilized less effi- / than was L-homocysteine, having ine-sparing value of 68% and a methionine-sparing value of only 7%.

The methionine activity of L-homocysteine varied when evaluated in a cystine-adequate diet, being more efficiently utilized at low levels of intake. However, at every level fed, L-homocysteine was inferior to L-methionine in supporting growth. The presence of additional choline in the diet did not improve the performance of chicks fed L-homocysteine as a source of methionine. Performance of chicks fed DL-homocysteine as a source of total sulfur amino acids was equal to that of chicks fed DL-homocysteine as a source of methionine per se. The utilization of the D and L isomers of homocysteine appears to be limited by the methionine activity they can replace.

The results of these assays clearly indicate that young chicks fed a crystalline L-amino acid diet cannot efficiently convert either D- or L-homocysteine to methionine via transmethylation.

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Differential Effects of Stress on Release of Thyroid-Stimulating Hormone in ' and Old Male Rats[1, 2] (40009)

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Age-related decreases in the responsiveness of the hypothalamo-hypophyseal system to several kinds of stimuli have been reported in the rat. Old female rats release less luteinizing hormone (LH) and follicle-stimulating hormone (FSH) than young female rats in response to ovariectomy (1, 2) and to progesterone treatment of estrogen-primed ovariectomized rats (3). Similarly, castration and ether stress result in release of less LH and FSH in old than in young male rats (2, 4). In response to acute cold exposure, old male rats release less thyroid-stimulating hormone (TSH) than young males (Huang, Chen and Meites, unpublished). Since all the previously employed stimuli resulted in increased release of anterior pituitary hormones, it was of interest to determine if a centrally mediated inhibition of TSH release changes with age. Acute stress has been shown to result in a rapid decrease in serum TSH in rats (5). It was the purpose of this study to compare the effect of acute stress on TSH release in young and old male rats.

Materials and methods. Male Sprague-Dawley rats were purchased from Charles River, Madison, Wis. at 7 months of age and maintained in our animal quarters until 18-20 months old. For comparison, 2- to 3-month-old male Sprague-rats were maintained in our lat for 1 month before experimentatio mals were housed in a temperature(and light(14 hr daily)-controlled ro were given Purina Rat Chow an *ad libitum*.

In Experiment I, serial blood san ml) were obtained by orbital sinu ture under light ether anesthesia 6, 12, and 24 hr after an initial b In Experiment II, animals were by ether exposure for 15 min. Blo ples were taken 1, 15, 30, and 60 n the initial ether exposure. In Exp III, physical restraint was accon by taping animals to test tube rac min. Blood samples were taken light ether anesthesia before and and 60 min after onset of restrain Serum samples from all experimer stored at −20° until assayed for the double radioimmunoassay met scribed in the NIAMDD kit. Seru values are expressed in terms of NI TSH-RP-1. Serum thyroxine (T_4) sayed by the Nichols Institute values are expressed as microgram per 100 ml of serum in terms of standard provided. The significance ferences between bleedings within age group was determined by an variance and Student-Newman-K (6). Differences between old anc rats at any time were determined dent's t test. The level of significa sen was $P < 0.05$.

Results. In Experiment I (Table rial bleeding in young males resul 67% decrease in serum TSH at 6 80% decrease at 12 hr, and a 48% r below zero time at 24 hr. In ol serial bleeding resulted in no ch serum TSH at 6 hr, a 33% decrea

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ır. and a 31% reduction at 24 hr. Serum T_4 was initially lower in old than in young nales. Serum T_4 in young males followed pattern similar to serum TSH, being lepressed at 6, 12, and 24 hr as compared vith the initial value. In old males, serum T_4 levels were correlated with TSH levels :xcept at 2 hr when serum TSH was high ınd serum T_4 was low.

Acute ether exposure in young males Fig. 1) resulted in a significant decrease n serum TSH at 30 and 60 min after nitial ether exposure. No change in serum TSH occurred in old males during the ame time period. In response to 5 min of physical restraint (Fig. 2), serum TSH in young males decreased 65% by 5 min after the onset of restraint and remained low through 60 min. In old males, physical restraint had no significant effect on serum TSH, although serum levels tended to decrease with time.

Discussion. These results demonstrate that, in response to several kinds of stress, serum TSH decreases more rapidly and to a greater extent in young than in old male rats. Since acute stress is known to inhibit TSH secretion in rats (5, 7, 8), it appears hat the central mechanism that mediates TSH inhibition is less sensitive in old than n young male rats. These findings are onsistent with the observation of an age-elated decrease in ability of the hypothalamus to inhibit prolactin secretion tonically (9, 10). The inability of old males to exhibit a normal TSH response to stress is imilar to the report of Riegle and Meites 4) that stress-induced LH release does ıot occur in old male rats. In contrast, here appears to be little age-related change in secretion of adenocorticoptropin (ACTH) in response to chronic stress (11).

The degree of TSH inhibition in young

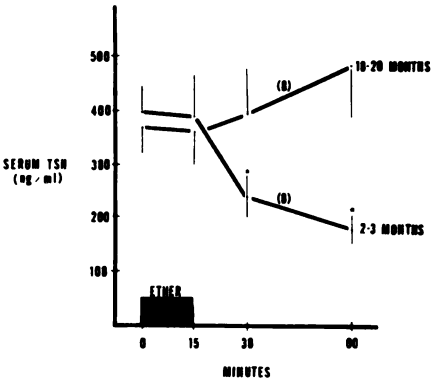

FIG. 1. Effect of 15 min of ether exposure on serum TSH in young and old male rats. Star indicates significant difference from initial sample. Numbers within parentheses indicate number of animals per determination. Solid bar indicates time of ether exposure.

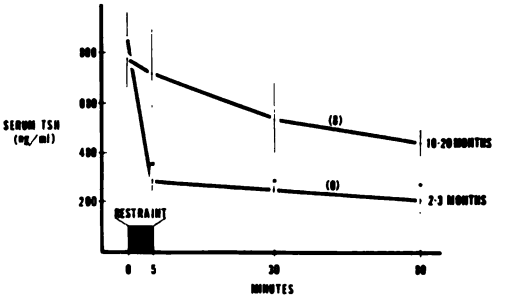

FIG. 2. Effect of 5 min of restraint stress on serum TSH in young and old male rats. Star indicates significant difference from initial sample. Numbers within parentheses indicate number of animals per determination. Solid bar indicates time of restraint.

TABLE I. EFFECT OF SERIAL BLEEDING ON SERUM TSH AND T_4 IN YOUNG AND OLD MALE RATS

	Hours after initial bleeding				
Age[a]	0	2	6	12	24
erum TSH (ng/ml)					
3–4 months	994 ± 159	775 ± 123	329 ± 68[b]	198 ± 55[b]	513 ± 68[b]
18 months	651 ± 70	790 ± 61	621 ± 75	433 ± 75[b]	448 ± 49[b]
erum T_4 (μg/100 ml)					
3–4 months	5.2 ± 0.2	4.9 ± 0.3	2.9 ± 0.3[b]	3.1 ± 0.2[b]	2.6 ± 0.2[b]
18 months	4.2 ± 0.3	3.3 ± 0.3[b]	4.7 ± 0.7	2.4 ± 0.3[b]	2.8 ± 0.2[b]

[a] Twelve or thirteen animals per determination.
[b] Difference from initial sample is significant ($P < 0.05$).

males was less in response to ether than to restraint stress. Acute ether exposure resulted in a 39% decrease in serum TSH by 30 min, which is similar to that reported by Fukuda *et al.* (12) and Männistö *et al.* (13). In contrast, physical restraint in young males resulted in a 65% decrease in serum TSH by 5 min. Mueller *et al.* (8) reported a 46% decrease in serum TSH after 5 min of restraint stress in rats killed by decapitation. The animals subjected to restraint stress were bled under light ether anesthesia. Thus the magnitude of restraint-induced TSH inhibition probably reflects the sum of both ether and physical restraint and could account for the larger decrease in serum TSH observed in the present study. In old males, physical restraint was also more effective than ether in decreasing TSH, although both stresses were less effective in old than in young rats.

Basal levels of serum TSH in old males tended to be lower than TSH levels in young males in this and in a previous report (10). The large variance in basal levels of serum TSH in both old and young males prevented these differences from being significant. Since serum T_4 also was lower in old than in young males, the age-related trend toward a decrease in basal levels of serum TSH appears to result from changes at the hypothalamo-hypophyseal level rather than to inhibitory feedback from T_4.

Since stresses have been reported to increase the turnover of catecholamines (14–16) and indoleamines (8, 17–19) in the hypothalamus of rats, the possibility exists that alterations in the ability of the autonomic nervous system to mediate a stress response occur with aging. Recently, we reported evidence for a decrease in DA turnover in the medial basal hypothalamus of old male rats (9), and there is considerable evidence that DA can inhibit TSH release. Thus the DA agonists, apomorphine and piribedil, have been reported to decrease basal serum TSH levels in male rats (20), and apomorphine blocked cold-induced TSH release (21). Therefore the decreased DA turnover in old male rats may be partially responsible for the lack of inhibition of TSH sponse to stress.

Restraint and electrical shock also reported to increase serotonergic a in several brain regions, includin hypothalamus, of young rats (8, 17 In old male rats, hypothalamic ser turnover appears to be elevated (10). serotonin influences on TSH releas in dispute (8, 22–25), it is difficult to its possible effects on TSH secretion stress. However, restraint stress has observed to increase hypothalamic tonin turnover and at the same ti depress serum TSH levels in mal (8). However, the relation of seroto the results observed here on TSH r in old male rats cannot be explair present.

Glucocorticoids have been shown hibit the secretion of TSH (7) anc have a role in the stress-induced inh of TSH secretion in old male rats. (11) reported a slight decrease in th ity of adrenals to secrete corticoster response to ACTH in old male rats apparent decrease in sensitivity c adrenals of old male rats could acco part for the absence of the stress-in TSH decrease. Further work is nec to determine which of these mech is responsible for the decline in capa the hypothalamo-hypophyseal syste old male rats to regulate TSH secret response to stress.

Summary. In response to the str serial bleeding, ether exposure, or cal restraint, serum TSH was dep more quickly and to a greater ext young than in old male rats. These suggest that the hypothalamo-hyp seal mechanisms which mediate inh of TSH release are less sensitive than in young male rats.

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TABLE II. LOCAL AND SYSTEMIC REACTIONS IN SEVEN ADULTS WHO WERE VACCINATED WITH POLYVALENT PNEUMOCOCCAL VACCINE INITIALLY AND AGAIN 13 MONTHS LATER

	Initial vaccination						Revaccination					
	No. with reaction on day						No. with reaction on day					
Reaction	0	1	2	3	4	5	0	1	2	3	4	5
Local												
Soreness	6	6	3	2			6	6	5	3	2	1
Erythema		1	1				2	7	4	3	1	
Induration							6	6	5	3	1	1
Systemic												
Chills							1	2				
Temperature												
Fever (max., 0)												
<99°F	6	6	7	6	7	7	4	5	6	5	6	5
99-99.9°F		1					3	2	1	1		
Not taken	1			1						1	1	2

and revaccinated at the same time periods used above with either of two additional lots of vaccine.

Infants. Table III shows that infants 6–24 months of age, who were given a single 0.25-ml dose of Lot 561/C-D194 12-valent pneumococcal vaccine, produced less antibody than did adults and showed varied serologic responses to the different types as well. Importantly, however, at least 50% of the infants did show a significant antibody increase to 10 of the 12 capsular types. The highest postvaccination antibody titers were against types 3, 4, 8, 9, and 56, and the lowest responses were against types 6, 12, 14, and 23. There were no substantial differences in responses in the 6- to 12-month age group compared with the 13- to 24-month age group. Limitations in the volumes of available sera precluded tests of sera from all the individuals against all the pneumococcal capsular types in the vaccine.

Infants 3–5 months of age (Study 378) who received Lot 561/C-D194 vaccine were revaccinated 6 months later. The results are shown in Table IV. The antibody increases on primary immunization were considerably less for most of the capsular types than seen in the older infants (Table III) and they declined rapidly for those types in which the best antibody responses had occurred. In addition, the geometric mean antibody titers following vaccination were generally low. In contrast to adults (see above), there was a substantially greater-fold increase in mean titer on revaccination, and this response was greatest for those serotypes in which there had been the highest initial response.

Infants in a second group (Study 378D), who had been vaccinated at 3 months of age, were revaccinated 21 months later. Blood samples had not been taken to measure antibody responses to the initial vaccination, but the titers of 21 months were roughly in the same range as for the children in Study 378 following the first vaccine dose. In contrast with the children in Study 378, who were given their booster dose at 9–11 months of age, these children showed responses with very high titer against all capsular types.

The infants showed very mild local reactions limited mostly to erythema and possible soreness in a portion of the group. In contrast to adults, there were no marked local reactions on revaccination in infants, and this finding was probably due to the smaller amount of pneumococcal capsular antibody in these persons (see below) at the time of revaccination.

Relationship between pneumococcal antibody titer and reactions. The cause for local brachial and systemic reactions in persons given pneumococcal capsular vaccine is not known with certainty but appears to be due to local reaction between the injected antigen and circulating antibody at the site in which the vaccine was

TABLE III. ANTIBODY RESPONSE, ACCORDING TO AGE, ON PRIMARY IMMUNIZATION OF INFANTS AND CHILDREN WITH POLYVALENT PNEUMOCOCCAL POLYSACCHARIDE VACCINE (STUDY 378)

Capsular type	Blood sample time (months)	Age of recipient: 6–12 months[a] Twofold or greater increase (number/total)	6–12 months[a] Geometric mean	6–12 months[a] Mean increase (-fold)	13–24 months[a] Twofold or greater increase (number/total)	13–24 months[a] Geometric mean	13–24 months[a] Mean increase (-fold)
1	0		120			136	
	1	25/31 (81)[b]	372	3.1	13/16 (81)	479	3.5
3	0		95			132	
	1	29/31 (94)	2200	23.2	14/14 (100)	3448	26.1
4	0		535			773	
	1	18/26 (69)	2084	3.9	10/15 (67)	1998	2.6
6	0		25			43	
	1	12/24 (50)	48	1.9	5/16 (31)	57	1.3
8	0		64			157	
	1	30/32 (94)	1112	17.4	14/16 (88)	1335	8.5
9	0		172			275	
	1	26/30 (87)	892	5.2	12/17 (71)	1060	3.9
12	0		33			37	
	1	11/24 (46)	57	1.7	12/16 (75)	80	2.2
14	0		95			124	
	1	4/10 (40)	112	1.2	4/10 (40)	173	1.4
19	0		40			60	
	1	17/20 (85)	169	4.2	9/10 (90)	255	4.3
23	0		44			67	
	1	13/16 (81)	102	2.3	5/10 (50)	100	1.5
51	0		52			88	
	1	25/29 (86)	279	5.4	9/11 (82)	416	4.7
56	0		88			195	
	1	24/29 (83)	838	9.5	11/15 (73)	1326	6.8

[a] 12-valent, Lot 561/C-D194, vaccine was used.
[b] Number within parentheses indicates percentage of total.

given. Support of this concept was shown in the findings presented in Table V which records the clinical reactions in 20 persons who were given vacccine, but who had different amounts of circulating antibody against the homologous capsular types at the time of vaccination. While there was no precise correlation between amount of pneumococcal antibody and the appearance of induration, persons with a large amount of antibody showed a greater chance of having more severe of a reaction than did those with a smaller amount of antibody.

Discussion. The present study of 12-valent pneumococcal vaccine in adults has confirmed previously published data (1–7) showing the ability of the vaccine to induce specific antibodies against the homologous types. The duration of protective effect of the vaccine is presently unknown, but it has been shown in previous studies (2, 10) with different pneumococcal vaccines that antibody induced by the vaccine was present at one-fifth to one-half or more of its peak values 2 or 3 years after a single injection. In some individuals, abundant residual antibody persisted 8 years after vaccination. The present study in adult volunteers showed only minor decline in antibody 13 months after the initial vaccine dose, and there was no substantial booster response when a second dose of vaccine was given at that time. There was, however, substantial increase in the degree of local reaction at the injection site after the second dose of vaccine which resulted in development of erythema and palpable induration. The optimal time for revaccination to achieve meaningful elevations in titer with least clinical response remains to be determined.

The correlation between the total amount of circulating homologous anti-pneumococcal capsular antibodies against

TABLE IV. INITIAL AND REVACCINATION ANTIBODY RESPONSES IN INFANTS GIVEN POLYVALENT (12-TYPE) PNEUMOCOCCAL VACCINE (STUDIES 378 AND 378D)[a]

	Study 378 (infants, 3-5 months)				Study 378D (infants, 3 months)			
Capsular type	Blood sample time (months)	Twofold or greater increase (number/total)	Geometric mean titer	Mean increase (-fold)	Blood sample time (months)	Twofold or greater increase (number/total)	Geometric mean titer	Mean increase (-fold)
1	0[b]		32		–[b]			
	1	4/8 (50)[c]	63	2.0	–			
	6[b]	1/8 (13)	42	1.3	21[b]		110	9.2
	7	6/8 (75)	118	3.7	22	30/31	1014	
3	0[b]		45		–[b]			
	1	5/5 (100)	525	11.7	–			
	6[b]	4/5 (80)	132	2.9	21[b]		136	
	7	5/5 (100)	386	8.6	22	30/31	3330	24.4
6	0[b]		20		–[b]			
	1	0.9 (0)	16	−1.3	–		40	
	6[b]	1/9 (10)	21	1.0	21[b]		198	
	7	0/9 (0)	19	−1.1	22	24/24		5.0
8	0[b]		76		–[b]			
	1	4/4 (100)	881	11.6	–			
	6[b]	4/5 (80)	357	4.7	21[b]		236	
	7	5/5 (100)	867	11.4	22	30/31	2980	12.6
9	0[b]		24		–[b]			
	1	7/7 (100)	107	4.5	–			
	6[b]	5/5 (100)	92	3.9	21[b]		136	
	7	6/6 (100)	176	7.4	22	29/29	1575	11.5
12	0[b]		14		–[b]			
	1	1/7 (14)	14	1.0	–			
	6[b]	3/7 (43)	22	1.6	21[b]		48	
	7	3/7 (43)	24	1.7	22	28/30	317	6.6
14	0[b]		74		–[b]			
	1	1/8 (12)	78	1.1	–			
	6[b]	1/8 (12)	64	−1.2	21[b]		48	
	7	3/8 (38)	96	1.3	22	17/22	293	6.2
19	0		55		–[b]			
	1	0/5 (0)	71	1.3	–			
	6[b]	0/5 (0)	68	1.2	21[b]		155	
	7	4/6 (67)	277	5.1	22	24/26	837	5.4
51	0[b]		27		–[b]			
	1	4/6 (67)	66	2.4	–			
	6[b]	4/6 (67)	75	2.7	21		80	
	7	5/6 (83)	95	3.5	22	19/20	637	8.0
56	0[b]		81		–[b]			
	1	3/4 (75)	269	3.3	–			
	6[b]	1/2 (50)	132	1.6	21[b]		310	
	7	4/4 (100)	578	7.1	22	28/28	4083	13.2

[a] Types 4 and 23 responses omitted because of too small number of serologic tests run due to small volume of serum.

[b] Time when vaccine was given.

[c] Number within parentheses indicates percentage of total.

all the types in the vaccine and the reaction to the vaccine is strongly suggestive of antigen-antibody formation at the local site with attendant Arthus-like (11) pathologic responses. Such reactions, while self-limited and generally mild, do impose restrictions on revaccination at a time when no substantial increase in antibody can be achieved. The possibility for reducing the amount of antigen per dose of vaccine to minimize local reactions on re-vaccination needs to be studied.

As with meningococcal polysaccharide vaccines (12), infants responded less well to pneumococcal vaccine than did adults. This was especially true of 3-month-old

.E V. RELATIONSHIP BETWEEN AMOUNT OF ULATING PNEUMOCOCCAL ANTIBODIES AND REE OF LOCAL REACTION TO POLYVALENT EUMOCOCCAL CAPSULAR POLYSACCHARIDE VACCINES

Local reaction		Total antibody N for 12 types, at time of vaccination (μg/ml)
ject	Degree of reaction[a]	
7	Slight to none	1,786
1	Slight to none	2,034
1	Slight to none	2,184
1	Slight to none	2,639
1	Slight to none	6,776
1	Slight to none	8,194
1	Slight to none	15,504
1	Slight to none	19,725
1	Slight to none	21,514
7	Slight to none	34,143
oup		114,499
1	Mod. w/induration	14,389
1	Mod. w/induration	16,358
1	Mod. w/induration	23,446
7	Mod. w/induration	28,149
1	Mod. w/induration	30,774
1	Mod. w/induration	40,026
1	Mod. w/induration	40,054
1	Mod. w/induration	44,100
1	Mod. w/induration	62,751
7	Mod. w/induration	131,081
oup		431,128

. There were considerable differ- among the capsular types. Antibody d by the vaccine in early infancy to decline quite rapidly. There was ked advantage to booster immuniza- f infants at 9–11 months of age, ılly for those capsular types in a primary response had been ed. Maximal antibody production otained, however, when the children revaccinated at 2 years of age and d antibody responses against all that approached the titers achieved ılts. These findings provide a basis pe that the vaccine will prove useful ınts and young children in prevent- vere pneumococcal disease and bac- a in cases of asplenia or impaired function, as well as in preventing onia, otitis media, and meningitis nal children.

mary. Adult persons developed sub- l antibody increases against essentially all pneumococcal capsular types following injection of polyvalent pneumococcal vaccine containing 50 μg of each capsular polysaccharide per dose. Revaccination 13 months after the previous immunization did not evoke important further increases in antibodies and there was substantially greater local reaction at the injection site than when the previous dose was given. This finding appeared due to local reaction of antigens with circulating antibodies in the area of injection, since there was a correlation between the measured amount of circulating pneumococcal antibodies and the degree of reaction. Infants less than 2 years of age who were given a half-dose of vaccine generally responded poorly when compared with adults. In studies of 3- to 5-month-old infants, there was some increase in antibodies when a booster dose of vaccine was given 6 months after the first. Very high level antibody responses against all capsular types were obtained when revaccination was delayed until 2 years of age.

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ect of Penicillamine on the Proliferative Response of Human Lymphocytes (40011)

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nicillamine (D-pen), β,β-dimethyl e, is a drug which has been shown to arious biological functions. Among ire copper chelation (1), interaction ystine to form a mixed disulfide (2), ion of collagen fibril formation (3), ochemical antagonism of vitamin B-6 *vitro*, it inhibits poliovirus replication nd has been reported to suppress rmation of antigen- and mitogen- ated lymphocytes (6).

n has been shown to be an effective eutic agent in the treatment of rheu- l arthritis. The clinical response to ug is accompanied by a fall in titer of atoid factor and a decrease in im- complexes in serum and synovial fluid is possible that the clinical response en is the consequence of an effect of ent on the immune system. This study ndertaken to investigate the interac- f D-pen with human lymphocytes *in*

erials and methods. Cell cultures. nocytes were isolated from normal nized blood (20 units/ml) on a Ficoll- ue gradient. The cells were washed with Hank's balanced salt solution ;) and cultured at a density of 1 × lls/ml in RPMI 1640 supplemented ! m*M* glutamine and 20% fetal calf . The purity of the isolated mononu- cells was determined by differential of the stained cells, and was more 0%. Cell viability was tested by the ion of trypan blue, and 97% of the ere viable.

ontinuous culture of normal human ocytes (Associated Biomedic Sys- was maintained in RPMI 1640 supple- d with glutamine and 5-7% fetal calf

gen stimulation. Cell cultures were ated with phytohemagglutinin (PHA) ughs-Wellcome Co.), 5 μg/ml, con- canavalin A (Con A) (Difco Laboratories), 20 μg/ml, and pokeweed mitogen (Difco Laboratories), 30 μg/ml. All cultures were run in triplicate and were incubated for a total of 72 hr, unless otherwise indicated, at 37° in 5% CO_2. D-Pen was dissolved in HBSS and added to cell cultures at varying concentrations at indicated times. Unstimulated lymphocytes, treated as above, were used as controls.

Mixed lymphocyte cultures. Mononuclear cells were isolated and cultured at a density of 1.5×10^6 cells/ml, as above. The stimulator cells were incubated with mitomycin C, 25 μg/ml, for 25 min at 37°. The treated cultures were centrifuged, washed twice with HBSS, and resuspended in complete medium. Aliquots (1 ml) of stimulator and responder cells were mixed and incubated with various concentrations of D-pen for 144 hr at 37° and 5% CO_2.

DNA, RNA, and protein synthesis. DNA synthesis was measured by the addition of 0.5-1.5 μCi/ml of [^{3}H]thymidine (22 Ci/mmole, Schwartz/Mann) to cell cultures 18 hr before harvesting, unless otherwise indicated. The cultures were centrifuged, washed with cold HBSS, and placed in an ice bath. Cells were lysed with 1 ml of cold H_2O and lysates were precipitated by the addition of 2 ml of 10% trichloroacetic acid (TCA). The precipitates were collected on Whatman GF/C glass-fiber filters and washed with 5% TCA. The incorporated radioactivity was measured in a Beckman scintillation spectrometer.

RNA and protein synthesis was measured by incubation of 0.1-0.5 μCi/ml of [^{14}C]uridine (488 mCi/mmole, Schwartz/Mann) or [^{14}C]leucine (340 mCi/mmole, Schwartz/Mann) with the cell cultures for 3 hr at 37° in 5% CO_2, and incorporated radioactivity was measured as above. The rate of RNA and protein synthesis was measured as describe previously (5).

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E rosette formation. The ability of D-pen-treated and untreated lymphocytes to form rosettes with sheep red blood cells (SRBC) was determined by the method of Bentwich *et al.* (8). Mononuclear cells at a density of 5×10^6 cells/ml were incubated in HBSS with varying concentrations of D-pen for 2 hr at 37°. The drug was removed by centrifugation and washing. The washed cells were resuspended in HBSS at the above density. One-tenth milliliter of a 0.5% suspension of sheep erythrocytes in HBSS was added to 0.1 ml of lymphocyte suspension, followed by the addition of 0.02 ml of heat-inactivated AB serum which had been absorbed with SRBC. The cell suspensions were incubated at 37° for 5 min, centrifuged at 200*g* for 5 min, and counted in a hemacytometer. Lymphocytes with three or more erythrocytes attached were considered rosettes.

Results. Effect of D-pen on DNA, RNA, and protein synthesis. D-Pen at concentrations ranging from 10 to 500 μg/ml was incubated for 3 and 6 hr with resting human lymphocytes and with human lymphocytes maintained in continuous culture. The drug had no effect on DNA, RNA, or protein synthesis.

Effect of D-pen on mitogen-stimulated lymphocytes. Addition of D-pen to lymphocyte cultures simultaneously with PHA, Con A, or pokeweed mitogen resulted in inhibition of transformation. This inhibition was partial, dose dependent, and maximal at 100 μg/ml (Table I). At this concentration, DNA synthesis was inhibited by 50% in PHA- and Con A-stimulated cultures, and by 40% when pokeweed was used as the mitogen. It had no effect on the number of surviving cells in the unstimulated control cultures.

TABLE I. EFFECT OF D-PEN ON DNA SYNTHESIS OF MITOGEN-STIMULATED LYMPHOCYTES.

D-Pen (μg/ml)	Counts per minute of [^{3}H]thymidine/10^6 cells at 72 hr		
	PHA	Con A	Pokeweed
0	14,000	18,000	10,000
25	13,000	15,500	9,200
50	9,500	13,000	6,200
100	6,800	8,700	6,000
200	7,000	8,500	5,800
400	7,400	9,000	6,200

Incubation of the cells with D-pen for 3 hr, followed by removal of the drug by centrifugation and washing prior to mitogen stimulation, resulted in a 20% inhibition of DNA synthesis.

Effect of D-pen on mixed lymphocyte cultures. Addition of D-pen to one-way mixed lymphocyte cultures resulted in inhibition of DNA synthesis (Fig. 1), which was maximal (60%) at a concentration of 100 μg/ml. Incubation of the stimulator cells with D-pen for 3 hr, followed by removal of the drug before addition of the responder cells, had little effect on DNA synthesis. Similar incubation of the responder cells resulted in a 40% inhibition of DNA synthesis (Fig. 2). The inhibitory effect was not augmented when *both* stimulator and responder cells were pretreated with the drug.

Effect of D-pen on E rosette-forming cells. Incubation of lymphocytes with D-pen for 2 hr and removal of the drug by centrifugation and washing before the addition of sheep red blood cells had no effect on the number of E rosettes.

Kinetics of D-pen inhibition of transformation. Since the proliferative response of lymphocytes to mitogen stimulation has been most extensively studied with PHA, the following experiments were performed with this mitogen.

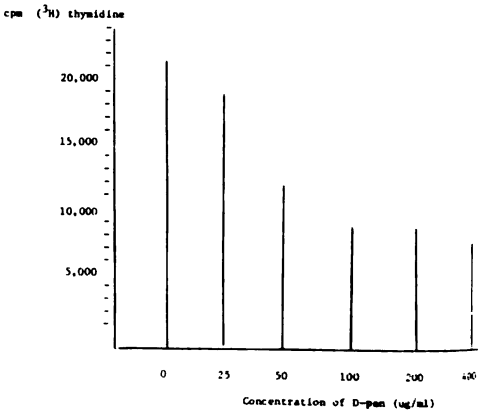
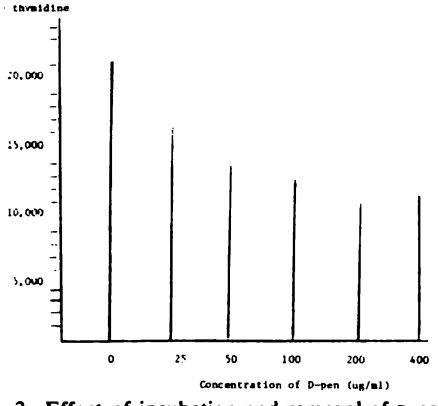


FIG. 1. Effect of D-pen on DNA synthesis in mixed lymphocyte cultures. Lymphocytes were isolated and cultured as described in the text. D-Pen was added at the concentrations shown to 2-ml aliquots of a mixture of stimulator and responder cells. Cultures were incubated for 144 hr at 37° in 5% CO_2. [^{3}H]Thymidine was added 18 hr before harvesting and DNA synthesis was determined.

effects of 100 and 300 μg/ml of D-
n protein, RNA, and DNA synthesis
[A-stimulated cells were determined
the drug and mitogen were added to
ocyte cultures simultaneously. Pro-
RNA, and DNA synthesis was mea-
in triplicate cultures at 3, 20, 48, and
poststimulation. D-Pen had no effect
NA, RNA, and protein synthesis of
imulated cells in the first 20 hr of
ation (Table II). At 48 and 72 hr,
tion of DNA synthesis was about 50%
here was a reduction in RNA and
in synthesis in parallel with the DNA
tion (Table II).



2. Effect of incubation and removal of D-pen
onder cells in mixed lymphocyte cultures. Lym-
es were isolated and cultured as described in
t. The responder cells were treated with concen-
s of D-pen as shown, and incubated for 3 hr at
5% CO_2. D-Pen was removed by centrifugation
shing. Cultures (1 ml) of stimulator cells were
to cultures (1 ml) of the D-pen-treated and
responder cells. Cultures were incubated for
at 37° in 5% CO_2. [^{3}H]Thymidine was added
efore harvesting and DNA synthesis was deter-

The effects of 100 and 300 μg/ml of D-pen on PHA-stimulated cells were determined at various time intervals. The drug was added simultaneously with PHA, and at 20 and 48 hr poststimulation. The addition of D-pen at zero time and 20 hr poststimulation resulted in a 50% inhibition of DNA synthesis; however, when the agent was added at 48 hr, it had no inhibitory effect (Table III).

Discussion. The above experiments show that D-pen inhibited lymphocyte responsiveness to nonspecific mitogen stimulation. In addition, the mixed lymphocyte reaction was also suppressed by the drug. The inhibition in both systems was partial and dose dependent (Table I, Fig. 1). These findings are in agreement with those reported by Schumacher *et al.* on the effect of D-pen on mitogen- and antigen-stimulated lymphocytes (6).

D-Pen had no effect on DNA, RNA, or protein synthesis in resting lymphocytes or lymphocytes maintained in continuous culture. We conclude, therefore, that the inhibitory effect of this agent is not due to

TABLE III. EFFECT OF D-PEN ADDED AT VARYING TIME PERIODS ON DNA SYNTHESIS IN PHA-STIMULATED LYMPHOCYTES.

Time of addition of D-pen (hr)	D-Pen (μg/ml)	Counts per minute of [^{3}H]thymidine/10^6 cells at 72 hr
0	0	24,000
	100	11,000
	300	12,000
20	0	25,000
	100	12,000
	300	11,500
48	0	23,500
	100	25,000
	300	24,000

ABLE II. EFFECT OF D-PEN ADDED SIMULTANEOUSLY WITH PHA ON MACROMOLECULAR SYNTHESIS MEASURED AT VARIOUS TIME PERIODS.

molecule	D-Pen (μg/ml)	Counts per minute/10^6 cells			
		3 hr	20 hr	48 hr	72 hr
otein	0	3,000	10,000	16,000	17,000
	100	3,400	9,500	10,000	11,000
	300	2,800	9,800	11,000	11,000
NA	0	4,500	16,000	28,000	26,000
	100	4,200	17,000	15,000	14,000
	300	4,500	15,000	16,000	15,000
NA	0	1,100	1,000	6,500	15,500
	100	1,000	1,200	3,200	8,000
	300	950	1,100	3,600	7,500

nonspecific inhibition of synthesis of those macromolecules.

The same degree of inhibition was produced when D-pen was added simultaneously with PHA or at 20 hr poststimulation, when the cells were fully committed to the proliferative state (Table III). [PHA stimulation of lymphocytes at 37° is not reversible after 6 hr (9).] The inhibitory effect, therefore, does not appear to be due to competition with or alteration of mitogen receptor sites on the cell surface.

The absence of effect of D-pen on lymphocyte surface antigens is indicated by the failure of the agent to inhibit rosette formation with sheep erythrocytes. In the mixed lymphocytes cultures, preincubation of the responder cells with D-pen resulted in inhibition of DNA synthesis (Fig. 2), while the preincubation of the stimulator cells had no effect. This also indicates that a nonspecific effect on lymphocyte surface antigens was not produced by the drug since that would have produced a similar degree of inhibition in both responder and stimulator cells.

In PHA-stimulated lymphocytes an increase in the rate of RNA and protein synthesis is evident shortly after stimulation (10, 11), and it becomes maximal in 24–48 hr. This increase in macromolecular synthesis is a prerequisite for the initiation of DNA replication, which commences approximately 20 hr poststimulation (12). D-Pen did not inhibit the induction of the increased rate of RNA and protein synthesis following PHA stimulation, as evidenced by the lack of effect of the drug on RNA and protein synthesis during the first 20 hr (Table II). The inhibitory effect of D-pen on lymphocyte transformation was evident only when the drug was present in the first 20 hr of culture. The inhibitory effect appeared to be related to some process(es) prior to the start of the replicative phase. DNA synthesis, which does not begin until about 20 hr poststimulation, is maximal in 48–72 hr (13). D-Pen inhibited DNA synthesis only when it was present during the first 20 hr after stimulation, even though it had no effect on the enhanced rate of RNA and protein synthesis during that period. When DNA synthesis was maximal, inhibition was not produced by the additio pen (Table III). It is possible that may exert its inhibitory effect by bl the signal for initiation of DNA repl or that it may partially inhibit the act of enzymes required for this proces ther studies are in progress to mo cisely define the mechanism of the tion.

Summary. Mitogen-stimulated human lymphocytes were exposed to icillamine (D-pen) at various concent and different time intervals. D-Pen inl lymphocyte response to mitogen stim and was also inhibitory to the mixe phocyte reaction. The inhibition was to be partial and dose dependent. Th bition of lymphocyte transformatio not due to nonspecific inhibition of p RNA, or DNA synthesis. The dr inhibitory only when it was present the first 20 hr of culture. The inh effect seemed to be related to some p prior to the initiation of the repl phase. It is suggested that D-pen ma the signal for the initiation of DNA r tion, or partially inhibit the activat enzymes required for the above proc

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centimeter	cm	milliosmole	mOsm
counts per minute	cpm	minute	min
cubic centimeter	cm^3	molal (concentration)	*m*
Curie	Ci	molar (concentration)	*M*
degree Celsius (Centigrade)	-°	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	diam	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	*N*
inch	in.	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
liter	spell out	second	sec
meter	m	specific activity	sp act
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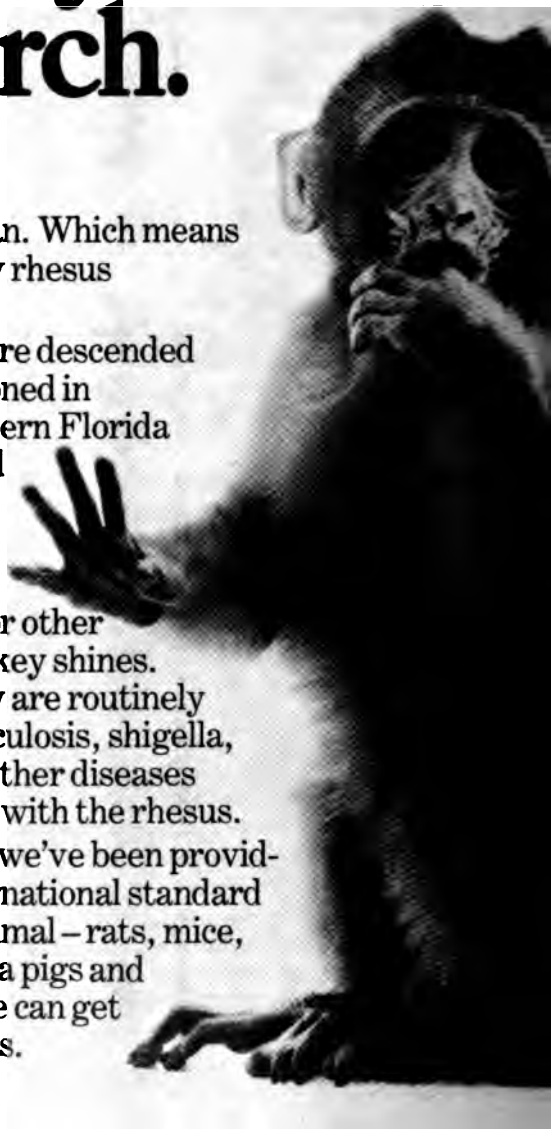

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TABLE OF CONTENTS

BIOCHEMISTRY

the Lysine Residues of Monellin	R. W. Morris, R. H. Cagan, R. E. Martenson, G. Deibler	194
Dependent Differences of Renal α-Galactosid-Galactosidase Activities in Two Highly Inbred hinese Hamsters with Spontaneous Diabetes	A. Y. Chang	319

ENDOCRINOLOGY

tin Stimulates Estrogen Production by Femin-nal Neoplastic Cells	V. S. Fang, N. Furuhashi, O. Gomez	159
ım Chloride on Adrenocortical Function in the	J. J. Jacobs	163
cose on Glucagon Secretion by the Anolian ncreas	W. B. Rhoten	180
d Hormone Levels in Sera of Juvenile Female onkeys	P. Hill, S. A. Tepper, D. Kritchevsky	231
Somatostatin and LH-RH after Hypophysec-lyper- or Hypo-Thyroidism, and during Anes-:ats	R. Fernandez-Durango, A. Arimura, J. Fishback, A. V. Schally	235
rculating Immunoreactive Human Parathyroid Comparison with a 1-34 N-Terminal Synthetic	C. Desplan, A. Jullienne, M. S. Moukhtar, D. Raulais, P. Rivaille, G. Milhaud	241

GROWTH AND DEVELOPMENT

nhibitor Activity in Mouse Uteri during Early and Delayed Implantation	D. Dabich, M. S. Owens	175
Development of Pulmonary Antioxidant En-he Rat	J. Yam, L. Frank, R. J. Roberts	293

HEMATOLOGY

f Action of Testosterone on Hemopoiesis *in* n Diffusion Chambers	A. Porcellini, A. Fontebuoni, G. Grilli, G. Lucarelli	184
tween Human Hemoglobin Variants and He-S	J. Kumpati, T. H. J. Huisman, G. D. Efremov	250
:xtran Sulfate in Increasing the CFU^{-}_{e} Concen-Dog Blood	W. M. Ross, M. Koerbling, W. Nothdurft, T. M. Fliedner	301

IMMUNOLOGY

link IgG, IgA, and IgM	J. E. Coe, R. E. Race	289
y and Paralytogenicity of Antigens Solubilized I_b Malignant Lymphocytes	R. E. Morris, W. H. Murphy	330

MICROBIOLOGY

Inhibits Antiviral and Growth Inhibitory Ac-Human Interferon	M. Degré	253

NUTRITION

:ation during a Developing Zinc Deficiency	E. D. Brown, W. Chan, J. C. Smith, Jr.	211
alnutrition on the Concentration of IgA, Lyso-ylase, and Aminopeptidase in Children's Tears	R. R. Watson, M. A. Reyes, D. N. McMurray	215

Influence of Dietary Fat and Carbohydrate on Food Intake, Body Weight and Body Fat of Adult Dogs — D. R. ROMSOS, M. J. HORNSHUH, G. A. LEVEILLE 278

Adipose Tissue Cholesterol Storage: The Effect of Essential Fatty Acid Deficiency — B. R. KRAUSE, S. Q. ALAM, A. D. HARTMAN 297

ONCOLOGY

Definition of the Cell Cycle Segment with Special Sensitivity to Vinblastine — A. M. CHIRIFE, G. P. STUDZINSKI 206

Elevated Thymidine Phosphorylase Activity in the Plasma and Ascitic Fluids of Tumor-Bearing Animals — J. L. PAULY, N. S. PAOLINI, R. L. EBARB, M. J. GERMAIN 262

PATHOLOGICAL PHYSIOLOGY

Effects of the Oral Converting Enzyme Inhibitor (SQ 14225) on One-Kidney Hypertension in the Dog — B. E. WATKINS, J. O. DAVIS, R. H. FREEMAN, G. A. STEPHENS, J. M. DEFORREST 245

PHARMACOLOGY

Glutathione-S-Transferase Activity in the Rabbit Nephron: Segmental Localization in Isolated Tubules and Formation of Thiol Adducts of Ethacrynic Acid — L. G. FINE, E. J. GOLDSTEIN, W. TRIZNA, L. ROXMARYN, I. M. ARIAS 189

Dual Excitatory Effect of Acetylcholine in the Mouse Vas Deferens — C. LINDAMOOD, III, S. M. JOHNSON, W. W. FLEMING 200

Effects of Fenfluramine and Norfenfluramine on Brain Serotonin Metabolism in Rats — R. W. FULLER, H. D. SNODDY, S. K. HEMRICK 202

PHYSIOLOGY

Effects of Ionophore A23187 and External Calcium Concentrations on Renal Gluconeogenesis — P. A. MENNES, J. YATES, S. KLAHR 168

Effects of β-Adrenergic Amines on Frog Gastric Mucosa — J. M. FARMER, G. CARRASQUER, T. L. HOLLOMAN, M. SCHWARTZ 256

Distribution Kinetics of Urea Between Plasma and Red Blood Cells of Normal Human Blood — M. JEEVANANDAM, C. L. LONG, J. M. KINNEY 282

Alterations in Bile Flow and Na^+-K^+ Biliary Excretion Induced by Theophylline and Ethacrynic Acid — R. G. KNODELL 306

REPRODUCTIVE PHYSIOLOGY

Blastokinin Secretion, Ovarian Activity, and Embryo Survival after Melengestrol Acetate in Rabbits — E. F. BOSTWICK, J. H. BRITT 220

VIROLOGY

Replication of Vesicular Stomatitis Virus Facilitated in Nonpermissive Cells by Early Functions of Shope Fibroma Virus — N. A. CROUCH 225

Radioimmunoassay of Measles Virus Antigen and Antibody in SSPE Brain Tissue — B. FORGHANI, N. J. SCHMIDT, E. H. LENNETTE 268

Differentiation of Serum Antibodies to Herpesvirus Types 1 and 2 by Radioimmunoassay — W. R. PATTERSON, W. E. RAWLS, K. O. SMITH 273

An *In Vitro* Transformation Assay for SiSV-1 and HL23V Using Feline Embryonic Fibroblasts — P. D. MARKHAM, S. Z. SALAHUDDIN, W. DODGE, R. E. GALLAGHER 312

An Unusually High Divalent Cation Requirement for Attachment of West Nile Virus to Primary Chick Embryo Cells — O. R. EYLAR, C. L. WISSEMAN, JR. 322

Epstein-Barr Virus Infection of Cryopreserved Umbilical Cord Blood Lymphocytes — T. C. SHOPE, M. K. SELL, S. T. SMITH, W. D. PETERSON, JR., C. S. STULBERG 326

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 159-162 (1978)

Prolactin Stimulates Estrogen Production by Feminizing Adrenal Neoplastic Cells (40012)

VICTOR S. FANG, NOBUAKI FURUHASHI, AND OFELIA GOMEZ

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luction. We have recently reported ıblishment of a clonal line of hu- ithelial cells derived from a femin- drenal neoplastic gland (FANG) ıignated them Fang-8 cells (1). In ıbculture, Fang-8 cells maintained ːific function of estrogen synthesis. ormal adrenal functions are under ıulatory control of adrenocortico- (ACTH) and possibly other pitui- ˙mones, we have carried out exper- to study the response of Fang-8 possible stimulation by anterior ˙ hormones in culture.

ials and methods. The cell culture nent was kept at 37°, 100% humid- CO_2–95% air. For replating, we ɾed 5–10 × 10^4 cells to each new 3003 petri dish (100 × 20 mm) and them in 6–10 ml of regular me- Matalon modified Eagle's medium taining 10% each of calf and fetal a]. To maintain the culture, me- as changed every 3 or 4 days. For entation, fresh medium was used ɯginning of experiments. If serum- ːdium was used, the culture was to 24 hr or a shorter period of t the end of each experiment, the ere harvested and washed three ˙ith normal saline before cellular was determined by the method of *t al.* (3).

gens secreted into medium were ːd by radioimmunoassay tech- l) with or without Sephadex LH-20 separation (5) for estrone (E_1) and l-17β (E_2). Fang-8 cells did not estriol (E_3) (1). When [^{14}C]acetate ɭed to medium as precursor, the ive estrogenic steroids secreted ːdium were extracted alternately er and 1 *N* NaOH twice and then d by the thin-layer chromato- graphic procedure (6). The identification of radioactive estrogens was achieved by precipitation with antisera specific to each steroid.

Pituitary hormones included human prolactin (hPRL) (73-4-27) which was generously donated by Dr. Henry Friesen, Manitoba, Canada; Cortrosyn (10 μg of β^{1-24}ACTH, equivalent to 1 USP unit), a product of Organon, Inc., West Orange, N.J.; and human GH (HS-2002F), human LH (LER-960), human FSH (LER-1575C), human thyrotropin (TSH-HS3), and rat PRL (I-1), all kindly supplied by NIAMDD, NIH, Bethesda, Md.

Results. Figure 1 shows the cell growth and estrogen production rates for the whole period of one subculture. In regular medium containing sera, cells started exponential growth on the third day after replating, as indicated by cellular protein. On the eighth day of subculture, cell growth began to decline and deceased cells floated in the medium. Cell growth was arrested completely after the 11th day, unless the surviving cells were immediately replated. Total estrogen production was correlated with cell protein during the subculture period. However, the E_2/E_1 ratio increased with the time of culture, from 0.33 on the second day to 2.6 on the eighth day. The synthesized estrogens were apparently secreted into medium constantly, as estrogen content inside the cells was always less than 0.1 pg/μg of cell protein, whereas medium content of estrogens might range from 2.8 to 8.8 pg/μg in 24 hr as calculated from data in Figs. 1A and B. When Fang-8 cells were cultured in serum-free medium for 24 hr, the cell growth was arrested and estrogen production was not halted, but was drastically reduced: The medium total estrogen content was 0.5–1.7 pg/μg (Fig. 1C). The

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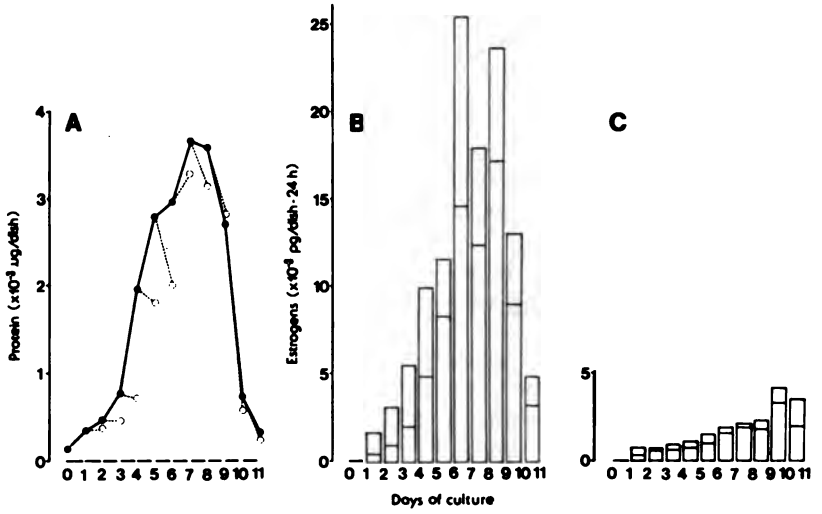

FIG. 1. (A) The growth curves of Fang-8 cells. Equal numbers of cells were replated to total 40 dishes on Day 0 and cultured in regular medium containing sera. From Day 1 on, four dishes were randomly taken daily during the following 10 days for 24-hr culture in 6 ml of fresh medium per dish: two dishes with regular medium containing sera (solid lines and circles) and the other two dishes with serum-free medium (dotted lines and open circles). The cells were harvested for protein determination. (B) The 24-hr production of E_1 (upper bar) and E_2 (lower bar) in medium, when cells were cultured in regular medium containing sera. (C) The 24-hr production of E_1 (upper bar) and E_2 (lower bar) in medium, when cells were cultured in serum-free medium. The values are the averaged means of two dishes.

E_2/E_1 ratio started at 0.35 on the second day and increased to 6.5 on the eighth day.

Fang-8 cells were able to synthesize radioactive estrogens from [^{14}C]acetate. In one experiment, Fang-8 cells on the fifth day after replating were cultured in regular medium containing sera and 1 μCi of [^{14}C]acetate per dish for various periods of time. The radioactivity incorporated into E_1 and E_2 increased with the time of exposure of Fang-8 cells to the isotope and amounted to 0.39 and 2.55 nCi at the end of 24-hr culture, respectively. The isolated radioactive E_1 and E_2 could be precipitated with excessive antisera coupled with a second antibody, up to 75 and 79%, respectively.

In regular medium containing sera, Fang-8 cells failed to be stimulated for estrogen synthesis by any single anterior pituitary hormone. However, using Fang-8 cells at their stage of exponential growth and cultured in serum-free medium, we could demonstrate a stimulatory effect of human PRL (1 μg/dish), as shown by the results in Table I. ACTH up to 10 μg, human GH up to 5 μg, and rat PRL up to 10 μg per dish failed to show a similarly stimulatory effect. Equivalent amounts of other human anterior pituitary hormones were ineffective. In a separate experiment, we further found that the stimulatory effect of human PRL on both E_1 and E_2 production by Fang-8 cells was a dose-dependent phenomenon (Table II). No similar dose-response relationship could be demonstrated with ACTH, hGH, and

TABLE 1. EFFECT OF PITUITARY HORMONES ON ESTROGEN PRODUCTION BY FANG-8 CELLS CULTURED IN SERUM-FREE MEDIUM.[a]

Hormone	Dose (µg/dish)	Cell protein (µg/dish)	Total estrogens in medium (pg/µg of protein in 24 hr)
None (control)	–	1185	0.58
hPRL	1	1250	2.10
ACTH	10	1206	0.78
hGH	2	1191	0.82
hLH	1	1185	0.59
hFSH	1	1178	0.62
hTSH	1	1200	0.69
rPRL	1	1210	0.55

[a] Mean value of two dishes for 24-hr culture.

TABLE II. RELATIONSHIP BETWEEN DOSE OF HUMAN PRL AND ESTROGENS SECRETED BY FANG-8 CELLS INTO SERUM-FREE MEDIUM IN 24 hr.[a]

Dose (μg/dish)	Cell protein (μg/dish)	E_1(pg/μg of protein)	P[b]	E_2 (pg/μg of protein)	P[b]
0 (control)	1445 ± 55	0.14 ± 0.04		0.34 ± 0.05	
0.05	1430 ± 90	0.15 ± 0.05	NS	0.38 ± 0.06	NS
0.1	1440 ± 69	0.38 ± 0.14	<0.01	0.53 ± 0.12	<0.05
0.2	1480 ± 23	0.58 ± 0.06	<0.001	0.95 ± 0.15	<0.001
1.0	1466 ± 20	0.55 ± 0.10	<0.001	1.20 ± 0.16	<0.001

[a] Values are means ± 1 SD for four dishes.
[b] Compared with control group by Student's *t* test.

rPRL. The data in both tables also indicate that cell protein did not increase after the hormone treatment. The E_2/E_1 ratio remained constant after human PRL treatment (Table II). Thus, the action of human PRL on estrogen biosynthesis seemed to be specific, probably to affect selectively the steroidal biosynthesis by a mechanism involving some important enzymes but not the interconversion between E_1 and E_2.

When the time courses of estrogen production by Fang-8 cells cultured in serum-free medium with and without human PRL were compared, the response to the stimulatory effect of human PRL was found to be rapid and continuous (Fig. 2). By 3 hr of culture, the difference in medium E_2 content between PRL-stimulated and control cultures was significant ($P < 0.01$). At the end of 24 hr, human PRL-stimulated cells secreted about three times the E_2 of the unstimulated culture. However, the E_1 production was stimulated to a lesser degree by human PRL.

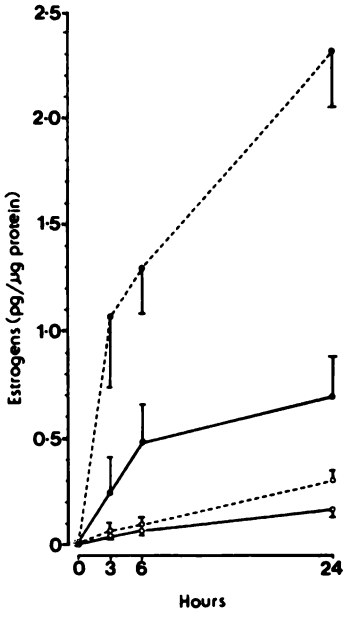

FIG. 2. Time courses of E_1 (open circles) and E_2 (solid circles) production by Fang-8 cells cultured in serum-free medium containing 0.2 μg of human PRL per dish (dotted lines) or no PRL as control (solid lines). The values are the means of four dishes and the vertical bars represent 1 SD.

Discussion. We were not totally surprised to notice that ACTH was ineffective in stimulating estrogen production by Fang-8 cells, as many adrenal tumors had failed to respond to ACTH (7). Millington *et al.* (8) recently reported that in primary tissue culture of a feminizing adrenocortical carcinoma, both ACTH and PRL stimulated the synthesis of estrogens and GH, LH, and ACTH were more effective than PRL in stimulating androgen synthesis. It is conceivable that being a clonal line, Fang-8 cells are a more specific type of cell culture than the mixed type of primary culture used by Millington *et al.* Furthermore, the difference between the two cultures could be accounted for if the two tumors were of entirely different types which nevertheless shared the characteristic of estrogen production. An earlier report by Mathur *et al.* (9) described another feminizing adrenocortical carcinoma which synthesized only estrone and Δ^5-steroid sulfates *in vitro* but not estradiol-17β. There were several scattered reports published since the review by Gabrilove *et al.* (10), describing feminizing adrenal tumors. The steroidal and gonadotropin production by these tumors were obviously heterologous. The steroidogenic property of Fang-8 cells resembled that of the BeWo and Jar lines of pure trophoblastic cells

derived from human choriocarcinomas, as described by Pattillo *et al.* (11). The trophoblastic cells could synthesize estrogens from androstenedione and dehydroepiandrosterone, probably not from acetate.

The adrenal gland is one of the target organs of prolactin, as suggested by receptor study (12). There is no unequivocal evidence for secretion of estrogens by a normal adrenal gland (13). There is also only suggestive evidence that prolactin may alter sex steroidgenic pathways (14). Current efforts to define the relationship between prolactin and steroidogenesis are less successful. The results from our experiments clearly demonstrated that human prolactin had a stimulatory effect on estrogen synthesis by Fang-8 cells. This finding strongly indicates that such an action by prolactin may exist under pathological conditions. Vermeulen *et al.* (15) recently reported that a supraphysiological prolactin level might directly stimulate the adrenal cortex for increased production of DHEA-S and androgen. It is conceivable that the feminizing adrenal cortex cells have the capacity to utilize these steroids for estrogen biosynthesis subsequent to the stimulation of prolactin. The lack of effect of rat prolactin and human growth hormone may involve hormone receptor specificity and could be further investigated.

It should be noted, however, that human prolactin stimulated estrogen biosynthesis by Fang-8 cells when they were cultured with serum-free medium, which restricted cell growth and function (Fig. 1). It seems true that in order to demonstrate the stimulatory effect of a hormone in culture, the cells should be in a hormone-depleted state which will sensitize the cellular response. An excellent precedent was the stimulatory effect of thyroid hormones on the growth of GH_1 cells, which could not be documented, unless cells were cultured with medium depleted of thyroid hormone (16).

Summary. Using Fang-8 cells c in serum-free medium, we could demonstrate a stimulatory effect of prolactin on estrogen biosynthesis concomitant stimulation of cell The enzymic mechanism involved been defined. Human ACTH, rat tin, and other human anterior p hormones were ineffective. This may bear some relevance to the pa cal action of prolactin on the adren for sex steroidal biosynthesis.

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Effect of Lithium Chloride on Adrenocortical Function in the Rat[1] (40013)

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ium salts are well-accepted thera- agents for the treatment of various disorders, particularly for the alle- of manic depression. During the cade, use of lithium has grown so that interest has focused on its of action as well as its possible ce on other biological systems. Cur- evidence supports a mechanism by lithium modifies various aspects tral biogenic amine metabolism (1,

siderable data have accumulated demonstrate an important, al- not fully understood, involvement tral biogenic amines in the modula- f the pituitary-adrenal gland axis Experimental manipulation of brain levels has been shown to alter pitui- cretion of ACTH and thus adreno- l hormone release. Since lithium ent has been associated with al- amine levels, it is reasonable to that adrenal gland function may cted as well. The few reports which ith the effect of lithium on adrenal n are difficult to interpret (7-10).

order to more fully ascertain the which lithium may have, the influ- f chronic lithium chloride treatment enal cortical function in the male examined.

rials and methods. Adult male e-Dawley rats (Charles River ng Laboratories, Inc., Mass.) weigh- proximately 200 g were maintained nt-controlled (fluorescent illumina- ghts on 0400 hr, lights off 1800 hr), ditioned (22-24°) rooms with food ter available *ad libitum*. They were individually and allowed to accli- mate to room conditions for at least 10 days prior to the experiments.

The first study was designed to examine the effect of chronic lithium chloride treatment on the rhythmic pituitary-adrenal function. Two groups of rats were used; one received lithium chloride (3 meq/kg body wt), orally, once a day (0800-1000 hr) for 2 weeks, and the other group was similarly treated with isotonic NaCl. On the day following the last drug injection, blood samples were obtained for studies of diurnal variation in plasma corticosterone (B) concentrations between 0800 and 0900 hr and 1600 and 1700 hr while the rats were in the resting state. Strict measures were employed to avoid disturbing the rats before blood sampling. No one was allowed in the animal quarters for at least 12 hr before sampling. Particular care was taken to ensure that each blood sample was obtained within 3 min after each "non-stressed" rat was removed from its cage. Each rat was then carried to an anteroom and immediately etherized; jugular blood (0.5-1.0 ml) was withdrawn into heparinized syringes. The "stress" response to ether inhalation was assessed at the same time. After the first blood sample was taken as described, the anesthetized rats were placed in individual cages in the anteroom for a 15-min period beginning with their first exposure to ether vapors. They were etherized a second time and another blood sample was collected.

The acute response to lithium was also studied. Groups of rats received one oral injection of LiCl (6 meq/kg body wt) or isotonic NaCl at 2, 12, or 24 hr prior to blood sampling. Basal B levels were measured at 0800-0900 hr.

The third segment of this investigation examined the effect of dexamethasone-21-phosphate on plasma B levels in rats chronically treated with LiCl or NaCl.

study was supported by a grant from the chlieder Educational Foundation, New Or- uisiana.

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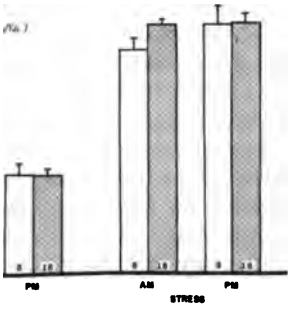
or diluted dexamethasone-
Decadron, Merck, Sharp
00 μg/kg body wt) was in-
s at midnight on the 14th
meq/kg) or NaCl treatment.
ther-stress blood samples
0800–0900 hr.
s collected in heparinized
lood was centrifuged at 4°
was separated and frozen
Plasma B was measured
by a modification of the
d by Kendall *et al.* (11).
levels were measured ac-
echnique of Amdisen (12).
was used for statistical
ta.
e-treated rats exhibited a
ariation in plasma B (AM,
00 ml; PM, 17.4 ± 2.2).
3 meq/kg body wt) admin-
male rats for 2 weeks did
AM–PM rhythm in plasma
it did cause a significant
ition in the morning (0800-
in comparison to saline-
ats (6.4 ± 1.4 vs 11.8 ±
e 1600-hr plasma hormone
ated animals (17.2 ± 1.3)
rom that of the controls
t the end of 2 weeks, the
weights (Δ = final –
newhat, but not signifi-
LiCl-treated group.
er anesthesia, the stress
ntrations rose dramatically



t of chronic LiCl or NaCl treat-
and "stress" plasma B levels in
essed as means ± SEM; number
n each column.

(Fig. 1) in all groups, as expected. In LiCl-treated rats, however, the 0800-hr plasma adrenal steroid concentration (44.0 ± 1.3) was elevated ($P < 0.05$) over that of its stress controls (39.4 ± 2.1); there was no comparable difference between the groups for the afternoon stress value.

The acute effect of LiCl administration on adrenocortical function is shown in Fig. 2. Young male rats given one oral dose of LiCl (6 meq/kg body wt) 2 hr prior to the 0800-hr blood draw responded with a marked elevation ($P < 0.05$) in plasma corticosterone level (32.9 ± 7.2) compared with NaCl-treated animals (17.5 ± 4.1). The difference in plasma hormone levels was evident, in spite of the presence of handling and injection stress factor which increased adrenocortical activity in both groups. When the time duration between drug or saline administration and blood sampling was extended to 12 and 24 hr, the corticosterone concentrations remained higher ($P < 0.05$) in the LiCl-treated animals. Note that the residual stress effect had dissipated by 12 hr after either injection.

In two separate assessments rats treated with LiCl or NaCl for 2 weeks were given one sc injection of either dexamethasone (100 μg/kg body wt) or saline 8 hr before sampling blood at 0800 hr (Fig. 3). The

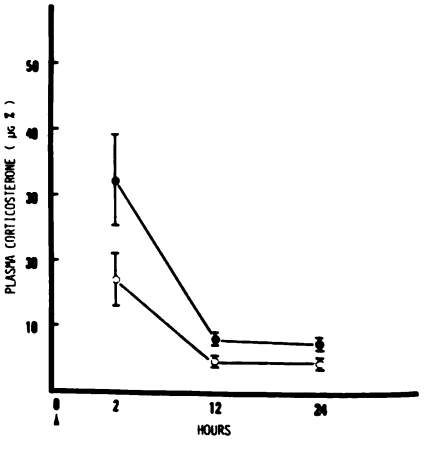
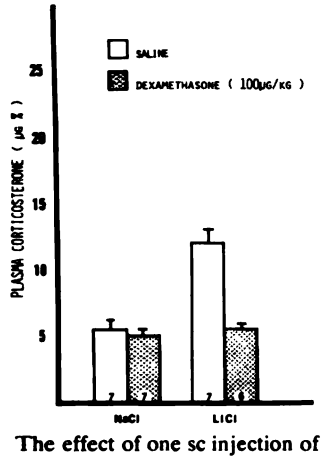


FIG. 2. Basal B secretion 2, 12, and 24 hr following an acute dose of LiCl (●) (6 meq/kg body wt) or NaCl (○). The injection time is indicated by the arrowhead; number of animals per group = 7; results expressed as means ± SEM.



3. The effect of one sc injection of dexameth-
100 µg/kg body wt) on plasma B levels in
onically treated with LiCl or NaCl. Results
ed as means ± SEM; number of animals
d in each column.

for one of the two experiments are
in Fig. 3. Notice that dexametha-
reatment did not cause an additional
ation in resting plasma B levels in
c NaCl-treated rats. One sc injection
) µg/kg body weight of dexametha-
however, did effectively suppress the
ed plasma steroid level typically
in long-term LiCl-treated animals
± 1.3 vs 5.5 ± 0.4; $P < 0.01$).

cussion. These data indicate that
ı affects the normal adrenocortical
ory pattern. In our laboratory the
l rhythm in adrenocortical secretion
e typical, i.e., plasma corticosterone
are lowest ("trough") at 0800 hr and
se steadily throughout the day to
their highest levels ("peak") at ap-
ıately 1600 hr. Although lithium ad-
ered daily for 2 weeks to male rats
ot abolish the fluctuation in plasma
ıl steroid levels (Fig. 1), it did con-
ly affect the rats in such a way as
use the early-morning plasma hor-
levels to be elevated midway be-
the normal trough and peak values.
vo separate occasions we measured
ı corticosterone in lithium-treated
: 4-hr intervals throughout a 24-hr
. The data from these studies con-
those illustrated in Fig. 1 in that
asma hormone level at each time
did not differ from that of saline-
treated control rats except for the 0800-hr value, which was elevated as shown in Fig. 1.

Few reports exist which deal with the influence of lithium on adrenocortical secretion. However, those which have been reported (7–10) are at variance with one another and difficult to interpret. Platman and Fieve (8) and Platman *et al.* (9) have presented evidence that the morning plasma cortisol levels are increased after lithium carbonate administration. Their data were collected from patients with manic depressive disease. On the other hand, in a study using normal healthy male volunteers, Halmi *et al.* (7) reported that lithium blunts the circadian variation of serum cortisol levels by lowering peak morning values. In still another investigation (10), lithium carbonate did not have any apparent effect on adrenal function in a group of patients in clinical remission. The discrepancies in these reports are most likely due to variations in the sampling population, dose of lithium used, duration of treatment, or time of blood sampling.

We attempted to minimize variables in these studies. Each male rat of the same age and weight was treated with an identical dose of lithium for the same period of time. Blood was sampled with as little disturbance to the rats as possible. We feel that our basal plasma corticosterone levels accurately reflect the efficacy of our sampling procedure since the "resting" rats display plasma hormone levels not unlike those of animals treated with a dose of dexamethasone sufficient to suppress adrenocortical function to basal levels (13). Initially the dosage and route of administration of LiCl were given special consideration. Various concentrations of LiCl (1.5, 3.0, 6.0, and 15.0 meq/kg body wt) were administered either ip or orally at 0800–1000 hr, daily, for 2 weeks. Since all rats receiving the two higher dosages of LiCl died within 4 days, the 3.0 meq/kg body weight dose was used in all experiments unless otherwise stated. This drug regimen resulted in lithium plasma levels in the range of 0.30 ± 0.030 meq/liter as measured 24 hr after the last injection.

No difference in results was observed between the two routes of administering the drug; therefore, in order to circumvent the possible nonspecific stressful effects due to peritoneal irritation, all drug treatments were administered orally in these studies.

Ether inhalation was used in one study to assess the effect of lithium on the functional integrity of the brain-pituitary-adrenal unit and of its ability to respond with increased ACTH release. The stress plasma corticosterone level in lithium-treated rats was greater ($P < 0.05$) than that of stressed saline-treated animals during the trough period of the diurnal cycle. There was no difference between the groups in the afternoon. Despite the increased response to ether in the lithium-treated rats, however, the "stress increment" (i.e., the difference between the respective basal and stress values) was not different between the two groups. In view of the uniform stress increments in experimental and control groups, it does not appear that 3 meq/kg body weight of lithium chloride compromised adrenal responsitivity to ACTH stimulation.

On the basis of evidence presented in this report, it seems unlikely that lithium chloride has a direct effect on the adrenal cortex. If it did, one would expect a nonspecific increase in adrenal corticosterone secretion at each time point examined, rather than only at the 0800-hr sampling period as shown here. Therefore, we presume that lithium administration results in a modulation of the brain-pituitary unit to increase ACTH release. Lending credence to this rationale are some preliminary data from this laboratory which demonstrate that lithium-treated hypophysectomized rats do not have elevated plasma corticosterone levels as do similarly treated intact controls.

Dexamethasone phosphate is a well-known inhibitor of ACTH release. It remains uncertain as to whether its locus of action is at the pituitary or brain level; one can muster evidence in support of either (14). In these studies dexamethasone (100 μg/kg) given 8 hr before blood sampling effectively inhibited the elevated corticosterone level seen with lithium treatment. This could indicate that the feedback effect of dexamethasone somehow interferes with the stimulatory influence of lithium. It is not unreasonable to assume that the interaction between the two drugs occurs at a neural level. As also suggested by other (7), the dexamethasone study provides further evidence that the adrenal cortex is not the primary site of action of lithium.

Since the clinical use of lithium is rapidly increasing, interest has focused on its exact locus and mode of action. In general most evidence indicates that lithium acts through induced changes in the metabolism of neural catecholamines and/or serotonin (15–18, 20, 21). O'Connell (22) has suggested that the commonly encountered side effects and behavioral consequences of lithium therapy are causally related, at least in part, to functional alterations in biogenic amine activity in limbic system structures particularly of the hypothalamus. Noradrenaline, dopamine, and serotonin are known to play important roles in hypothalamic control mechanisms as they relate to brain-pituitary-adrenal gland function (4–6, 23). Mukherjee *et al.* (19) reported that more lithium is selectively accumulated by hypothalamic tissue than by cortex, cerebellum, and other areas. It is likely then that the therapeutic effectiveness, side effects, and possible deleterious endocrine consequences of lithium treatment reflect an action by lithium at some hypothalamic or other limbic system locus.

In this study it is conceivable that lithium chloride may have produced a general stress reaction to which the adrenal gland responded with an increase in secretion of corticosterone. In addition, the possibility that lithium may have altered corticosterone disposal rates cannot be ruled out. More detailed pharmacological information is needed to support the hypothesis that the effects caused by lithium on the adrenal gland are probably mediated by modifications in the neurochemical circuits known to influence adrenocortical function.

Summary. The effect of lithium chloride treatment on adrenocortical function was

studied. Rats given lithium orally for 2 weeks demonstrated elevated AM "resting" plasma corticosterone levels compared to saline-treated controls. There was increased plasma corticosterone 2 hr after a single lithium injection. Dexamethasone blocked the lithium-stimulated adrenal response in chronically treated rats.

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Effects of Ionophore A23187 and External Calcium Concentrations on Renal Gluconeogenesis (40014)

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Since the initial suggestion of a close relationship between calcium and cyclic-3',5'-adenosine monophosphate by Rasmussen and Tenenhouse (1), it has become evident that calcium acts as a second messenger in the control of many diverse cellular processes (1-3). The nearly universal nature of this close relationship between calcium and cyclic AMP has been the subject of several recent reviews (2, 3). It appears that in the many systems which are regulated by calcium, cyclic AMP plays an important role as a modulator of that signal (2, 3). In certain systems calcium and cyclic AMP seem to act synergistically, in that cyclic AMP augments the initial calcium effect, where in other systems cyclic AMP appears to oppose the calcium effect (3).

The activation and modulation of renal gluconeogenesis by parathyroid hormone is a system in which calcium and cyclic AMP are closely related (4). Borle has shown a significant increase in radiocalcium efflux from kidney cells in tissue culture following the addition of parathyroid hormone (5). After the demonstration of calcium requirement for maximal rates of gluconeogenesis in the perfused rat liver by Hems *et al.* (6), many investigators have examined the role of extracellular calcium and hydrogen ion concentrations in the control of renal gluconeogenesis (7-17). Parathyroid hormone stimulates renal gluconeogenesis in the presence of external Ca^{2+} but not in its absence, despite a similar increase in cyclic AMP levels under both conditions (10, 15). In addition, prostaglandin E_1 has been shown to increase gluconeogenesis (18), an effect which is inhibited by removal of external Ca^{2+}. Again, cyclic AMP generation in response to PGE_1 was identical in the absence and presence of external Ca^{2+}.

Ionophorous antibiotics modify the transport of specific ions across biological membranes. The ionophore A23187 is a carboxylic acid antibiotic derived from *Streptomyces chartreusensis* that is specific for the transport of divalent cations across biologic membranes (19). A23187 specifically binds cations at neutral pH with the following relative affinities $Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$ (18). The ionophore A23187 has recently been demonstrated to activate many calcium-dependent biological processes, including fluid secretion in the insect salivary gland (20), DNA synthesis in sea urchin eggs (21), secretion and aggregation in platelets (22), and insulin secretion in pancreatic β cells (23).

The present studies were undertaken to examine the roles of calcium and cyclic AMP in renal gluconeogenesis using the ionophore A23187 in an effort to alter intracellular calcium concentrations independent of any effect on cyclic AMP concentrations.

Materials and methods. Preparation of renal cortical tubules. Rat renal cortical tubules were prepared by a modification of the method of Nagata and Rasmussen (10). Adult Holtzman rats weighing 200-300 g, fed a regular diet, and not fasted prior to study were stunned by a blow to the head. The abdominal aorta was exposed through a midline incision and the kidneys were perfused via the aorta with 10 ml of a calcium-free Krebs-Ringer phosphate buffer, pH 7.4, containing 40 mg% of collagenase (Sigma Corp., St. Louis, Mo.). Following the perfusion, the kidneys were removed rapidly and placed in ice-

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alcium-free Krebs-Ringer phosphate , pH 7.4. Cortical slices were ob- using a Stadie-Riggs microtome. The were then incubated at 37° in 10 ml same calcium-free buffer containing % of collagenase for 20–30 min in a)ff metabolic shaker at a rate of 60–70 tions per minute. The slices were using a mixture of O_2/CO_2 (95:5, Following this incubation the tubules filtered through two layers of fine al gauze and washed with 50 ml of n-free Krebs-Ringer phosphate, pH he suspension was then centrifuged for 90 sec at 4° in a Sorvall centrifuge el Rc2-B). The pellet was resuspended ml of the calcium-free Krebs-Ringer hate and the procedure was repeated he final preparation of tubules was ended in calcium-free Krebs-Ringer hate buffer.

rmination of glucose production. ie determination of glucose produc- 500-μl aliquot of the suspension of cortical tubules containing 4–6 mg tein was incubated in Krebs-Ringer iate buffer, pH 7.4, of the following composition (millimoles per liter): 30; K^+, 4; Mg^{2+}, 1.4; Cl^-, 136; PO_4^{2-}, his solution also contained 2% BSA. iments were carried out with no cal- Ca^{2+} was omitted and 0.1 mM EGTA dded), or with 0.1 or 1.0 m*M* Ca^{2+} itration in the incubation medium. Ca^{2+} was omitted, the Na^+ concen- of the Ringer's was 132 mmoles/ The final incubation volume was 1.0 l experiments were performed in 10- iconized Erlenmeyer flasks contain- her 10 m*M* α-ketoglutarate, 10 m*M* iine, 10 m*M* succinate, 10 m*M* lac- 0 m*M* glycerol, or no substrate. The s were incubated at 37° in a Dubnoff olic shaker at 50 oscillations per e and were constantly gassed using ure of O_2/CO_2 (95:5, v/v).

erimental flasks contained iono- A23187 at concentrations of 10^{-8} or *1*. Control flasks contained DMSO, same concentration as that present experimental flasks, since the iono- A23187 stock solution was made up ISO. Tubules were, therefore, incu- bated without substrate, with substrate and ionophore A23187, and with substrate and DMSO. After 60 min of incubation the reaction was stopped by boiling for 3 min. The suspension was then centrifuged at 3000*g* for 5 min and an aliquot was obtained for glucose determination. Glucose was assayed fluorimetrically using the hexokinase method (24). All measurements were done in duplicate. Protein was measured by the method of Lowry *et al.* (25).

Measurements of cyclic AMP. Suspensions of renal cortical tubules containing 4–6 mg of protein were incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing 10 m*M* glucose, 10^{-2} *M* theophylline, and 0.25% lyophilized bovine serum albumin. The calcium concentration was either 0, 0.1, or 1.0 m*M*. Experimental flasks contained ionophore A23187 at concentrations of 10^{-8} and 10^{-9} *M*, appropriate DMSO diluent, or parathyroid hormone, 1 unit/ml. Incubations were carried out for 20 min at 37° in a Dubnoff metabolic shaker using a gas mixture of CO_2/O_2 (5:95, v/v). The reaction was terminated by the addition of trichloroacetic acid. After ether extraction the suspension was centrifuged at 3000*g* for 5 min and the supernatant assayed for cyclic AMP. The pellet was placed into boiling 50 m*M* sodium acetate/acetic acid buffer, pH 4, for 10 min and homogenized by hand using a glass homogenizer. The homogenate was then centrifuged at 5000*g* for 20 min and the supernatant assayed for cyclic AMP. Cyclic AMP was determined by a modification of the method of Gilman (26). All measurements were done in duplicate. Similar experiments were carried out without theophylline in the medium and incubating for 10 min.

Reagents. [^{3}H]-Labeled cyclic-3′,5′-AMP was purchased from New England Nuclear (Boston, Mass.). Bovine parathyroid hormone synthetic 1-34, Lot No. 26013, was from Beckman. The ionophore, A23187, was a generous gift from Dr. Robert Hamill of the Eli Lilly Co., Indianapolis, Indiana. The ionophore was stored at 4°. Fresh solutions were prepared daily by adding 20 μl of DMSO/0.5 mg of ionophore and

bringing to a final volume of 1 ml with distilled water.

The results are expressed as means ± the standard error. Statistical analysis was performed using Student's *t* test for paired data.

Results. The effect of parathyroid hormone on glucose production by isolated rat renal cortical tubules is presented in Table I. In the presence of 0.1 and 0.25 m*M* external calcium, submaximal doses of parathyroid hormone stimulated significantly glucose production from α-ketoglutarate. Omission of calcium from the incubation medium (0.1 m*M* EGTA) abolished the stimulation of gluconeogenesis produced by parathyroid hormone. Glucose production was linear during the 60 min of incubation.

Table II presents the effect of ionophore A23187 on glucose production from α-ketoglutarate by isolated rat renal cortical tubules. The rates of glucose production from α-ketoglutarate at external Ca^{2+} concentrations of 1.0 m*M* (130.9 nmoles/mg of protein/60 min) are lower than those obtained by Nagata and Rasmussen (10) using an external Ca^{2+} concentration of 2.5 m*M* (179 ± 9 nmoles/mg of protein/hr). Ionophore A23187 (10^{-8} and 10^{-9} *M*) increased glucose production significantly ($P < 0.01$) in the presence of 10 m*M* α-ketoglutarate at external calcium concentrations of 0.1 m*M*. Increased ionophore concentrations, such as 10^{-6} *M*, inhibited glucose production at 0.1 m*M* external calcium concentrations. Omission of calcium from the incubation medium, which contained 2% BSA and 0.1 m*M* EGTA, decreased glucose production from α-ketoglutarate, and addition of ionophore, 10^{-8} and 10^{-9} *M*, did not increase glucose production significantly. At external calcium concentrations of 1.0 m*M* and higher, 10^{-8} and 10^{-9} *M* ionophore did not increase glucose production from α-ketoglutarate.

To explore further the role of external calcium present in bovine serum albumin (15), additional experiments were performed using dialyzed BSA. The BSA was dialyzed at 4° for 72 hr against a phosphate Ringer's solution containing no calcium. Table III shows the effects of ionophore on glucose production by isolated rat renal cortical tubules when dialyzed bovine serum albumin was used. In the absence of external calcium, 10^{-9} *M* ionophore did not significantly increase glucose production as compared to control flasks containing diluent (DMSO) at the same concen-

TABLE I. EFFECT OF PARATHYROID HORMONE ON GLUCOSE PRODUCTION BY ISOLATED RAT RENAL CORTICAL TUBULES.

	Glucose production (nmoles/mg of protein/60 min)[a]		
	No external Ca^{2+}	0.1 mM External Ca^{2+}	0.25 mM External Ca^{2+}
No substrate	20.9 ± 2.5	24.6 ± 4.6	34.4 ± 1.4
10 m*M* α-Ketoglutarate	78.8 ± 3.7	93.2 ± 2.7	101.2 ± 3.2
Parathyroid hormone, 1 unit/ml	77.5 ± 4.2	121.2 ± 3.7	145.0 ± 5.8

[a] Values are the means ± SE of four experiments.

TABLE II. EFFECT OF IONOPHORE A23187 ON GLUCOSE PRODUCTION BY ISOLATED RAT RENAL CORTICAL TUBULES.

	Glucose production (nmoles/mg of protein/60 min)		
	No external Ca^{2+}	0.1 m*M* External Ca^{2+}	1.0 m*M* External Ca^{2+}
No substrate	22.3 ± 2.1[a]	30.3 ± 1.8	46.5 ± 5.0
α-Kg + 10^{-8} DMSO diluent	76.3 ± 7.1	113.3 ± 10.9	130.9 ± 9.3
α-Kg + 10^{-8} *M* ionophore	81.5 ± 9.5	130.0 ± 12.6*	113.2 ± 4.4
α-Kg + 10^{-9} DMSO diluent	66.4 ± 6.6	115.4 ± 10.7	117.7 ± 4.1
α-Kg + 10^{-9} *M* ionophore	66.1 ± 7.3	136.4 ± 10.2*	115.6 ± 7.6

[a] Values are the means ± SE of four experiments in the cases of zero and 1.0 m*M* external Ca^{2+} and seven experiments with 0.1 mM external Ca^{2+}.

* Values significantly different from those obtained with α-Kg + DMSO 10^{-8} ($P < 0.01$).

tration. On the other hand, using dialyzed BSA and a 0.1 m*M* external calcium concentration, ionophore produced a significant ($P < 0.01$) increase in glucose production as compared to the values obtained using diluent alone.

Table IV depicts the effect of 10^{-8} *M* ionophore on glucose production from a variety of substrates at an external Ca^{2+} concentration of 0.1 m*M*. Under these conditions ionophore resulted in a significant increase in glucose production ($P < 0.05$) from α-ketoglutarate, glutamine, succinate, and lactate. Ionophore did not increase glucose production when glycerol was used as a substrate.

Since many compounds have been shown to stimulate gluconeogenesis by increasing cyclic AMP levels, we studied the effect of ionophore A23187 on cyclic AMP levels in isolated renal cortical tubules. Table V presents the results of such studies. Ionophore A23187 at concentrations of 10^{-8} and 10^{-9} *M* did not increase cyclic AMP production by renal tubules at external Ca^{2+} concentrations of 0.1 or 1 m*M*. Omission of Ca^{2+} from the external medium did not alter the results. On the other hand, 1 unit/ml of parathyroid hormone increased cyclic AMP production significantly both in the absence and presence of external Ca^{2+}.

Similar experiments (results not shown) were carried out without theophylline in the incubation medium at 0 (0.1 m*M* EGTA), 0.1, and 1 m*M* external Ca^{2+} concentrations. Ionophore did not increase cyclic AMP production under these conditions.

Additional experiments were performed to evaluate the effects of ionophore alone, cyclic AMP alone, and the two drugs in combination on glucose production by isolated rat renal cortical tubules. As shown in Table VI, both ionophore and cyclic AMP added alone increased glucose production significantly as compared to flasks containing only α-ketoglutarate. However, the combination of ionophore and cyclic AMP did not result in a further increase in glucose production as compared to the flasks containing either ionophore or cyclic AMP alone. These experiments would suggest that the effects of ionophore and cyclic AMP on glucose production are not additive.

Pentobarbital, a widely used inhibitor of calcium transport, completely abolished the increase in glucose production observed with ionophore A23187 at external calcium concentrations of 0.1 m*M*. Preincubation with 10^{-5} *M* pentobarbital for 20 min was required to observe this effect. The results of these experiments are shown in Table VII.

TABLE III. Effects of Ionophore A23187 on Glucose Production by Isolated Rat Renal Cortical Tubules.[a]

	Glucose production (nmoles/mg of protein/60 min)	
	No external Ca^{2+}	0.1 m*M* External Ca^{2+}
No substrate	10.9 ± 0.5	15.6 ± 3.3
α-Kg + 10^{-9} DMSO diluent	35.6 ± 4.8	60.4 ± 7.4
α-Kg + 10^{-9} *M* ionophore	30.3 ± 5.6	82.3 ± 8.1*

[a] These experiments were carried out using dialyzed bovine serum albumin (BSA). BSA was dialyzed at 4° for 72 hr against a phosphate Ringer's solution containing no calcium. The values are the means ± SE of six experiments.

* Values significantly different ($P < 0.01$) from α-Kg + 10^{-9} DMSO.

TABLE IV. Effect of Ionophore A23187 on Glucose Production by Isolated Rat Renal Cortical Tubules.[a]

Substrate	No. of experiments	No substrate	Substrate and DMSO	Substrate and ionophore
α-Ketoglutarate	7	30.3 ± 1.8	113.3 ± 10.9	130.0 ± 12.6*
Glutamine	5	35.6 ± 4.2	93.9 ± 9.6	110.7 ± 10.1*
Succinate	4	39.3 ± 3.6	94.9 ± 17.8	116.4 ± 21.9*
Lactate	4	36.9 ± 3.2	87.1 ± 2.9	95.0 ± 2.3*
Glycerol	4	26.7 ± 2.4	61.2 ± 7.6	57.9 ± 7.2

[a] Values are means ± SE. External Ca^{2+} concentration, 0.1 m*M*. Substrates used at 10 m*M* concentration.

* Values significantly different ($P < 0.05$) from those obtained with substrate + DMSO.

TABLE V. EFFECT OF IONOPHORE A23187 AND PARATHYROID HORMONE ON CYCLIC AMP PRODUCTION BY RAT RENAL CORTICAL TUBULES.

	Cyclic AMP (pmoles/mg of protein/20 min)		
	1.0 m*M* External Ca^{2+}	0.1 m*M* External Ca^{2+}	No external Ca^{2+} (0.1 m*M* EGTA)
Control	12.4 ± 1.8[a]	9.9 ± 1.9	15.1 ± 0.6
10^{-8} DMSO diluent	11.4 ± 0.7	9.1 ± 2.7	16.7 ± 0.3
10^{-8} *M* Ionophore	10.7 ± 1.2	9.5 ± 3.3	16.1 ± 0.5
Parathyroid hormone, 1 unit/ml	47.5 ± 4.1	44.7 ± 10.8	68.1 ± 11.0
10^{-9} DMSO diluent	12.6 ± 0.9	8.9 ± 2.5	17.2 ± 1.0
10^{-9} *M* Ionophore	10.0 ± 0.3	8.9 ± 2.4	14.2 ± 2.0

[a] Values are the means ± SE of four experiments.

TABLE VI. EFFECTS OF IONOPHORE A23187 AND CYCLIC AMP ON GLUCOSE PRODUCTION BY ISOLATED RAT RENAL CORTICAL TUBULES.[a]

	Glucose production (nmoles/mg of protein/60 min)
No substrate	21.6 ± 1.3
α-Kg + 10^{-9} DMSO	109.4 ± 7.4
α-Kg + 10^{-9} *M* ionophore	134.4 ± 8.1*
α-Kg + 5×10^{-4} *M* cyclic AMP	147.7 ± 6.5*
α-Kg + 10^{-9} *M* ionophore + 5×10^{-4} *M* cyclic AMP	134.4 ± 4.7

[a] Values are the means ± SE of four experiments. External Ca^{2+} concentration was 0.1 m*M*.

* Values significantly different ($P < 0.01$) as compared to those obtained with α-Kg + DMSO.

TABLE VII. EFFECT OF 10^{-5} *M* PENTOBARBITAL ON IONOPHORE-STIMULATED GLUCOSE PRODUCTION BY RENAL CORTICAL TUBULES.

	Glucose production (nmoles/mg of protein/30 min)			
	α-Kg	α-Kg + pentobarbital	α-Kg + ionophore	α-Kg + ionophore + pentobarbital
Experiment 1	24.6	23.9	33.7	22.8
Experiment 2	34.4	32.3	40.4	31.6
Experiment 3	30.2	28.9	44.4	33.3
Experiment 4	23.7	30.0	56.4	27.2
Mean ± SEM	28.2 ±2.5	28.8 ±1.8	43.7 ±4.8	28.7 ±2.4

Discussion. The results of the present experiments demonstrate that the divalent cation ionophore A23187 increases glucose production from a variety of substrates in isolated renal cortical tubules. The effects of ionophore on gluconeogenesis were present at low levels of extracellular calcium (0.1 m*M*). In the absence of external calcium or at concentrations of extracellular calcium in excess of 1.0 m*M*, the ionophore did not stimulate renal gluconeogenesis. The critical role of calcium in the control of renal gluconeogenesis has been documented previously (10, 15, 18). In the absence of extracellular calcium, glucose production is not increased by a variety of stimuli capable of increasing gluconeogenesis when Ca^{2+} is present. On the other hand, cyclic AMP levels have been shown to increase in response to several agents even when calcium is omitted from the incubation medium (15, 18).

The lack of stimulation of gluconeogenesis by the ionophore at extracellular calcium concentrations of 1.0 m*M* might be due to the development of high cytosolic calcium concentrations. The fact that high concentrations of ionophore (10^{-6} *M*), rather than stimulating, inhibited gluconeogenesis also suggests that at these concentrations the ionophore may markedly increase cytosolic Ca^{2+} even when the external Ca^{2+} concentration was 0.1 m*M*.

The ionophore A23187 increased glucose production from α-ketoglutarate, glutamine, succinate, and lactate, but not from glycerol, suggesting that the ionophore may modulate (via changes in cytoplasmic calcium) the activity of the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase. Previous studies by Roobol and Alleyne (27) have demonstrated this enzyme to be sensitive to changes in calcium concentration.

The increase in glucose production from α-ketoglutarate produced by the ionophore at external Ca^{2+} concentrations of 0.1 m*M* was completely abolished by pretreatment of the renal cortical tubules with 10^{-5} *M* pentobarbital. Pentobarbital has been shown to inhibit Ca^{2+} transport across

certain biological membranes (28). Thus, as calcium transport across the cell membrane into the cytosolic compartment was blocked, increases in glucose production did not occur using the ionophore, indicating that the ionophore most likely stimulated glucose production by altering cytosolic calcium concentrations.

Ionophore A23187 did not increase cyclic AMP levels in renal cortical tubules or slices at various calcium concentrations. The presence or absence of theophylline in the incubation medium did not alter this lack of effect. Glucose production was thus stimulated by the ionophore without an increase in cyclic AMP levels. Parathyroid hormone (4), prostaglandin E_1 (18), and catecholamines (29, 30) increase cyclic AMP levels and gluconeogenesis. In the case of both parathyroid hormone and prostaglandins, removal of external Ca^{2+} blocks the increased gluconeogenesis produced by these compounds without affecting cyclic AMP production. The ionophore in the insect salivary gland does not stimulate cyclic AMP levels (20), but in both the pancreatic islet cells (23) and in bone culture cells (31) it has been shown to cause an increase in cyclic AMP levels.

In the present studies, glucose production was stimulated by ionophore in the absence of an increase in cyclic AMP by a mechanism felt to be related to changes in cytosolic calcium concentrations. No additive effects on gluconeogenesis were observed when ionophore and exogenous cyclic AMP were added together (Table VI). At low levels of extracellular calcium, the ionophore stimulated glucose production presumably by increasing cytosolic calcium concentrations. This action was blocked by pretreatment with pentobarbital. At higher extracellular calcium concentrations, the ionophore did not stimulate gluconeogenesis, suggesting that there is an optimal concentration of cytosolic Ca^{2+} for gluconeogenesis and high Ca^{2+} concentration may be inhibitory of this process. Mechanisms designed to maintain cytosolic Ca^{2+} concentrations within narrow limits seem to be critical in the control of renal gluconeogenesis.

Summary. The divalent cation ionophore A23187 was found to increase glucose production from α-ketoglutarate, glutamine, succinate, and lactate, but not from glycerol in isolated rat renal cortical tubules at external Ca^{2+} concentrations of 0.1 m*M*. At an external Ca^{2+} concentration of 1.0 m*M* or in the absence of external Ca^{2+}, the ionophore did not increase glucose production from α-ketoglutarate. Ionophore A23187 did not increase cyclic AMP levels in renal cortical tubules. The increase in glucose production produced by ionophore A23187 was completely prevented by preincubation of the tubules with 10^{-5} *M* pentobarbital, a known inhibitor of calcium transport in biological systems. The experiments suggest that ionophore A23187 increases gluconeogenesis from a variety of substrates by altering the levels of cytosolic calcium without affecting the concentrations of cyclic AMP.

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sin-Like Inhibitor Activity in Mouse Uteri during Early Gestation and Delayed Implantation (40015)

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duction. The invasiveness occur- implantation sites in rodent uteri s that lytic mechanisms may be d in implantation. Support for the ation of proteinases, in particular, early embryonic development and ation is based on the following itions: (a) Maximum uterine fluid ytic activity, which is believed to e blastocyst attachment to the coincides with the initiation of ation (1); (b) trophoblasts seques- 3) and digest (4) particulate cellular l during the invasive phases of ation; (c) proteolytic activities may be involved in cellular diges- d/or cell surface transformation of ysts have been detected (5–7), and oteinase inhibitors administered *in* mpair implantation (8). The results asurements of proteinase inhibitor in uteri during early gestation pecies differences (9, 10). To estab- lationships between proteinase ac- ssociated with implanting embryos erine proteinase inhibitor activity, ements of the respective biochemi- ivities must be made on a single . The investigations in this report, re, pertain to quantification of tryp- inhibitor activity (TLI) in mouse luring early gestation and delayed ation.

rials and methods. Synchroniza- f ovulation and delayed implanta- The ovulation of virgin, female, Webster mice of approximately 8 of age was synchronized by injection J of FSH (Equinex; Ayherst Labs., .Y.) followed by 5 IU of chorionic tropin (Ayherst Labs., Inc., N.Y.) Following the latter injection, mice were mated and checked for copulation plugs the following morning, designated Day 1 of pregnancy. Control animals were treated with the same hormonal regimen, but not mated. Delayed implantation in some pregnant mice was induced by ovariectomy performed on the morning of Day 4 of pregnancy. These animals were treated with 1 mg of progesterone daily (12).

Preparation of uterine homogenates. After sacrificing mice by cervical dislocation, uteri were excised and placed into cold (4°) physiological saline until each experimental group was collected. All procedures during the preparation of the respective extracts were performed at 4°. Uteri were trimmed free of blood vessels and connective tissue, flushed with cold physiological saline to remove uterine fluid and unattached embryos, then weighed after blotting excess fluid. In cases where implantation had occurred (i.e., Day 6, 8, or 12 of pregnancy), the embryos were either left attached or gently removed from the uterine wall with forceps. The weighed uteri were minced well with scissors prior to homogenization (1 g wet weight/2 ml of 0.25 *M* sucrose in 0.05 *M* Tris-HCl buffer, pH 7.5) in a Potter-Elvehjem homogenizer, approximately 10 passes. The homogenate was centrifuged at 27,000*g* for 30 min. No inhibitor activity was found in the pellet before or after solubilization with various concentrations of Triton X-100. The supernatant solution (27,000*g*) contained inhibitor activity which did not sediment at 100,000*g* after 1 hr.

Fluorometric assay. Analysis of TLI activity in the uterine extracts was performed at 37° with a modified fluorometric kinetic assay (13). Each assay mixture contained: 0.20 ml of 0.0116 *M* BANA (Benzoyl-DL-arginine-β-naphthylamide,

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Schwarz/Mann, Orangeburg, N.Y.) dissolved in methyl cellosolve; 0.05 ml of a trypsin solution (sp act = 12,000 BAEE units/mg; Sigma Chemical Co., St. Louis, Mo.) containing 0.10 mg of trypsin/ml of 0.10 *M* phosphate-citrate buffer, pH 7.5 (assay buffer); aliquots of a 1:10 dilution of the 27,000*g* supernatant solution diluted with assay buffer; and sufficient assay buffer to adjust the final volume to 5.0 ml. The inhibitor was incubated with the enzyme at 37° for approximately 8 min before triggering the reaction with substrate. Hydrolysis of BANA was monitored with a Turner fluorometer, Model 111, equipped with thermospacers and attached to a recorder. A 7-60 primary filter and 50% interference filter were used in conjunction with a 2A-12 secondary filter.

A standard curve relating fluorescence measurements to β-naphthylamine concentration was used to determine the amount of BANA hydrolyzed. Pure β-naphthylamine, a white powder with a melting point of 110°, was obtained after numerous recrystallizations from water. One unit of inhibitor activity was defined as the amount of inhibitor required to inhibit the hydrolysis of 1 nmole of BANA/min. To obtain specific activities (units of inhibitor per milligram of protein), protein determinations were completed according to Lowry *et al.* (14).

Identical assays, but without trypsin, obviated possible interference by endogenous peptidases in the uterine extracts. Moreover, inhibition of trypsin by large amounts of albumin, as 500 μg (Fraction V, Sigma Chemical Co., St. Louis, Mo.), were considered and found to have no effect. Blood plasma was also analyzed for TLI activity to determine the extent to which possible blood contamination of homogenates might contribute to total inhibitor activity. The amount of contamination was ascertained by determining the amount of hemoglobin in homogenates compared to whole blood by the cyanomethemoglobin method (15) (Hycell, Inc., Houston, Tex.).

Results. Quantitative measurements of inhibitor activity. The specific activity of TLI in uterine extracts prepared at specific times of gestation is shown in Figs. 1 and 2. The histograms are based on the mean value for each group. Each individual experiment represented an analysis of an extract prepared from 5 to 10 uteri. Inhibitor activity due to blood contamination was found to be less than 2.6% of the total activity.

The elevated specific activity of TLI in uterine extracts prepared on Day 6 of pregnancy is different ($P \leq 0.05$) from values found for hormone-treated controls, Days 4 and 12 of normal pregnancy (Fig. 1), or Days 6 and 8 during implantation delay (Fig. 2). The data were analyzed by multiple comparison of means (16). Moreover, a P value of 0.055 was obtained when the specific activity of TLI of Day 6 pregnant uteri (Fig. 1) was compared to that of nontreated controls (Figs. 1 and 2) or Day 5 of implantation delay (Fig. 2).

One must consider the fact that embryos remaining *in situ* in uteri used to prepare uterine extracts on Day 6 of pregnancy (Fig. 1) might themselves contribute significantly to the observed increase in inhibitor activity relative to control samples. This is believed not to be the case for the

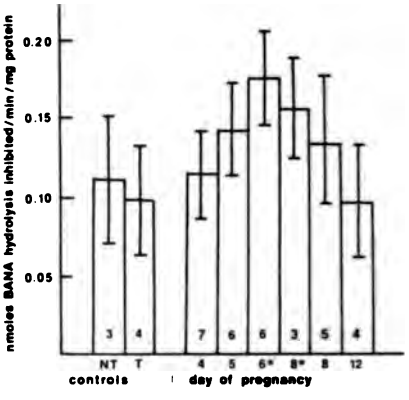
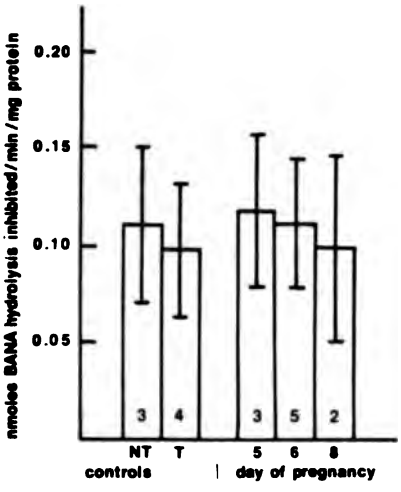


FIG. 1. Specific activities of tryptic-like inhibitor in uterine extracts prepared from nonpregnant or pregnant uteri. The control mice were either treated hormonally (T) to achieve cycle synchronization or not treated (NT). Numbers at the base of the histograms denote the number of independent experiments. The perpendicular lines at the top of each histogram indicate the 95% confidence limits. An asterisk beside the number indicating day of pregnancy denotes the fact that the embryos were not removed from these uteri.



2. Specific activities of tryptic-like inhibitor rine extracts prepared from nonpregnant or d implantation, pregnant uteri. The control s were either treated hormonally (T) to : cycle synchronization or not treated (NT). :rs at the base of histograms denote the r of independent experiments. The perpendic- ıes at the top of each histogram indicate the ɔnfidence limits.

/ing reasons. First, the embryos rep- : only a very small fraction of the mass of the Day 6 pregnant uterus 9). Second, one can estimate that ximately 10% of the total embryonic ın which could be present in a Day 6 e extract must be in the form of an tor, such as soybean trypsin inhibi- SBTI), to account for the increase. calculation is based on the following: e efficacy of a purified preparation of in the assay described in this report; ıe average number of embryos per ; (19); and an assumption that there 00-fold increase in embryonic protein g development between Days 5 and 6 :gnancy (17, 18). The calculated level ıthesis for a specific protein (approxi- y 10% in this case) is not usual, ıgh exceptions in specialized tissues e found (20–22). Finally, attempts in aboratory to identify an isoinhibitor ssible embryonic origin and different :ctrophoretic mobility from those in ı or extracts of control uteri were not ısful. Consequently, the increased tor activity appears to be the result of modulation of TLI levels by the uterus and/or transduction of serum proteins, among which are proteinase inhibitors, into uterine tissue. Reasons for serious consideration of the latter possibility are described below.

Effects of pH and temperature on inhibitor activity. Although acidification of uterine extracts to pH values of 6.0 or 5.0 prior to analysis did not result in loss of inhibitor activity, dropping the pH value to 3.9 led to complete loss of activity. Alternatively, a basic milieu (pH 8.5 or 10.5) had no effect on stability. Complete inactivation of inhibitor activity also resulted after heating extracts for 5 min at 60°. These results contrast with those obtained by Blackwood *et al.* (9) for rat uterine trypsin-like inhibitors, but correspond to the reported characteristics of serum (23–25) and oviductal fluid (26) trypsin-like inhibitors in terms of both pH and temperature labilities.

Discussion. On the basis of studies with a macromolecular dye and ^{125}I-labeled protein, Psychoyos (27) has suggested that the estrogen surge (28, 29) which occurs in rodents at implantation causes transudation of serum proteins into uterine tissue. Contributing to this effect may be estrogen from blastocysts (30). The presence of serum-like proteinase inhibitors in the reproductive tract has been reported in the case of oviductal fluid of both rabbits (26) and monkeys (31).

The following observations in this study appear to be in accord with the possibility of transudation and the presence of serum-like TLI activity in the extracts: (a) The time profile (Fig. 1) indicates maximum activity just subsequent to the estrogen surge (28, 29); (b) the temperature and acid pH lability of the inhibitor preparations is similar to the TLI activity in serum (23–25); and finally (c) the specific activities of TLI are similar in nonpregnant (control) and delayed implantation uteri. One cannot discount, however, the possibility that elevated inhibitor activity on Day 6 may be related, in part, to an inflammatory-like response of the uterus during the invasive stages of implantation.

Several physiological functions can be proposed for the TLI activity in the uterine extracts. (i) It may be necessary to balance proteinase and proteinase inhibitor activities of both embryo and uterine environment for implantation to occur. This is indicated by the fact that exogenous proteinase inhibitors administered *in utero* impair implantation (6), while addition of trypsin to mouse blastocyst cultures initiates further development *in vitro* (32). (ii) The uterine inhibitors may modulate the activity of trypsin-like proteinases of the blastocyst (6, 7) or uterine fluid (1). (iii) The inhibitors may control or restrict the activity of proteinases liberated by leukocytes which are found at implantation sites (33); without modulation of proteolytic activity, digestion of tissue beyond areas of implantation might also occur. Finally (iv) the inhibitors, like α_1-antitrypsin and α_2-macroglobulin, may act as antifibrinolytic agents (25, 34, 35) and prevent blood clotting in the capillary penetrated by the embryo during implantation.

Summary. Specific activities of trypticlike inhibitors (TLI) in 27,000*g* supernatant solutions of mouse uterine homogenates were determined for the following conditions: nonpregnant, pregnant, and delayed implantation. Quantification of inhibitors was achieved by a fluorometric kinetic assay. Inhibitor levels in uteri from Day 4 pregnant and from delayed implantation mice were the same as nonpregnant uterine control values. A significant increase in inhibitor activity (P = 0.05) was found in uteri from Day 6 pregnant mice. This increased activity returned to control levels by Day 12 of pregnancy. Possible functions of TLI in regulating proteolytic activity associated with early development and implantation are discussed.

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Effects of Glucose on Glucagon Secretion by the Anolian Splenic Pancreas (40016)

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Introduction. The clinical hallmark of human diabetes mellitus is hyperglycemia. Hyperglycemia is a distinctive characteristic of the American anole, *Anolis carolinensis* (1). An attenuated insulin secretory response to glucose is observed in the anole (2, 3), unborn mammals including man (4–7), and prediabetics and diabetics (8–10). Recently we presented evidence that the anole has fasting hyperglucagonemia relative to the prevailing level of blood glucose (11). The significance of glucagon in human diabetes is not established. Results which suggest that glucagon may be important in the mediation of diabetic hyperglycemia and the clinical course of the disease have been reported (12–14). Some morphological evidence has demonstrated a relative or absolute increase in the number of α cells in juvenile diabetes (15, 16). Approximately 50% of the endocrine cells in the anolian pancrease are α cells (1). Pancreatectomy in the α cell-rich lizards results in a rapid onset of hypoglycemia which persists for several days (17, 18). The present investigation was initiated in order to better characterize α cell regulation in this species.

Materials and methods. Experimental procedures. Two days prior to sacrifice, adult anoles were placed in an environmental chamber with 14 hr of light alternating with 10 hr of darkness. The temperature was 33° during the light cycle and 23° during the dark cycle. The splenic portion of the pancreas was removed from decapitated lizards. Each splenic pancreas was placed in Hanks' buffer, pH 7.3, and freed of extraneous tissue. The splenic pancreata were initially incubated for 30 min at 33 ± 0.5° in disposable cups for automatic analysis (W. Sarstedt, Inc., Princeton, N.J.) in 0.3 ml of incubation medium. The basic incubation m was a Krebs-Ringer-bicarbonate s containing 2.25 mg/ml of D-glucos tional Bureau of Standards, Washi D.C.), 0.5% (w/v) plasma albumin IV, Sigma Chemical Co., St. Louis, 500 kIU of aprotinin/0.3 ml (Sigma ical Co.), and, in milliequivalents pe Na, 141.5; chloride, 129.9; Ca, 5.8; bonate, 20.0; phosphate, 5.0; K, 5. Mg, 2.24.

The cups, each containing two pancreata, were placed in glass s tion vials and capped with serum stoppers. The vials were placed in a rocating shaker bath (100 strokes/n cm amplitude) and continuously with a humidified mixture of 95% CO_2 to maintain pH 7.3–7.4. Aft equilibration period the medium w moved by aspiration and 0.3 ml basic buffer was added (basal incub After 30 min, the medium was as off and medium containing variou centrations of D-glucose and, in cases, arginine (L-Arg HCl) was (experimental incubation). Incubatic continued for 60 min. The mediu aspirated off and the pancreata were and weighed. For dynamic studies 8 splenic pancreata were perifused scribed previously (2).

Radioimmunoassay of glucagon. ditions and characteristics of the gl radioimmunoassay have been de previously (11). Glucagon levels we termined with incubation medium appropriate dilutions (1:20 to 1:20 samples were assayed in duplicate dilutions which resulted in less tha displacement of tracer hormone. St curves were generated from medi corresponding dilutions. The porcir cagon standard was generously pr

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by Dr. Mary Root of the Eli Lilly Research Laboratories. The radiolabeled hormone, [^{125}I]glucagon, was diluted with assay buffer to yield a standard curve of suitable displacement (New England Nuclear, Cambridge, Mass., or Cambridge Nuclear, Billerica, Mass.). Glucagon antisera were kindly provided by Drs. Anthony S. Pagliara and Howard S. Tager. The characteristics of Dr. Tager's antisera have been described (19).

Results. The mean glucagon release during the basal incubation (2.25 mg/ml of glucose) for all experiments was 31.6 ± 5.1 pg/mg of splenic pancreas/min (N = 37). The range of secretion for all basal incubations was 2.1 to 141.5 pg/mg/min. Glucagon release during the basal incubation was not significantly different from the release with the same concentration of glucose during the experimental incubation of 60 min ($P > 0.05$ by unpaired t test). This result was observed in spite of the more narrow range of values for glucagon secretion during the experimental period (2.3 to 47.6 pg/mg/min).

A 56% decrease in the concentration of glucose (1.0 mg/ml) failed to evoke a significant change in the rate of glucagon release (Table I). The addition of arginine (10 mM) at the same concentration of glucose did not significantly alter the rate of release. A significant increase in glucagon release did occur when the glucose concentration was decreased to 0.5 mg/ml (Table I). In the presence of arginine, glucagon release was not significantly different from that obtained in its absence ($P > 0.4$ by unpaired t test).

The kinetics of glucagon secretion when splenic pancreata were perifused with the combination of 0.5 mg/ml of glucose and arginine (10 mM) are shown in Fig. 1. A striking increase in glucagon release occurred rapidly after changing to this medium. Enhanced release developed concomitantly with the fall in glucose concentration below 1.0 mg/ml. The peak of early release occurred between 4 and 8 min after the glucose level fell below 1.0 mg/ml. The mean maximal rate of secretion during the first 8 min was 1.02 ± 0.17 ng/mg of splenic pancreas/min.

A high concentration of glucose, 6 mg/ml, failed to significantly decrease gluca-

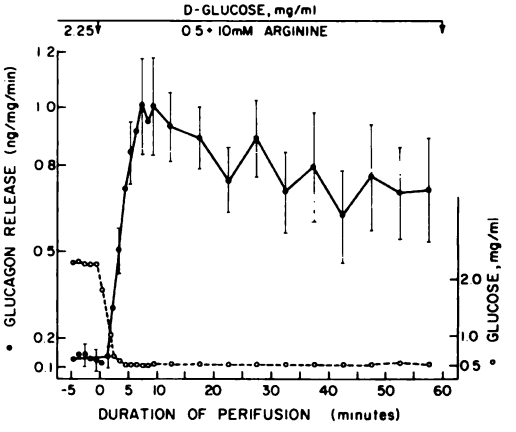

FIG. 1. Pattern of glucagon release from splenic pancreata perifused with 0.5 mg/ml of glucose and 10 mM arginine. The splenic pancreata were initially perifused with 2.25 mg/ml of glucose for 60 min. The medium was changed to one containing low glucose and arginine, and perifusion was continued for another 60 min. Time 0 is 2 min after changing to the new medium. Vertical bars represent the standard errors of the means. The number of observations was three.

TABLE I. GLUCAGON RELEASE FROM ANOLIAN SPLENIC PANCREATA INCUBATED WITH VARIOUS CONCENTRATIONS OF GLUCOSE.[a]

Basal incubation (2.25 mg/ml of glucose)	Experimental conditions		Number of observations	Statistical analysis
	Glucose (mg/ml)	Glucagon release		
32.0 ± 12.8	2.25	32.2 ± 8.3	6	NS
27.3 ± 4.9	1.0	49.2 ± 7.0	4	NS
29.2 ± 7.3	1.0 + 10 mM Arg	113.1 ± 37.7	6	NS
36.0 ± 20.4	0.5	217.9 ± 59.0	7	<0.005
20.2 ± 5.9	0.5 + 10 mM Arg	299.3 ± 90.3	7	<0.02
43.0 ± 13.0	6.0	15.4 ± 3.7	7	NS

[a] Results are expressed as picograms of porcine glucagon equivalents per milligram of splenic pancreas per minute ± SEM. Glucagon release under experimental conditions was compared with release during the basal incubation by means of the paired t test. A P value less than 0.05 was considered significant.

gon secretion (Table I). The range of release was 5.1 to 28.0 pg/mg of splenic pancreas/min.

Discussion. This study has shown that glucagon secretion by the anolian splenic pancreas is relatively insensitive to changes in the concentration of glucose. *Anolis carolinensis* has a normal fasting blood glucose level of about 2.2 mg/ml (1, 2, 20). In either relative or absolute terms, glucagon release in response to a low concentration of glucose is attenuated in the anolian α cell. Arginine does not correct this deficit. The anolian α cell responds rapidly when the level of glucose is severely depressed. This rapid onset of glucagon secretion is similar to that found in either perfused rat pancreas (21) or perifused isolated rat islets (22).

Studies with other experimental animals have shown that metabolic substrates other than glucose may have significant physiologic regulatory roles in islet cell function (23–25). The absence of a significant effect of arginine on glucagon release by the anole is in marked contrast to mammals, including man, where numerous studies have shown an increase in glucagon secretion in response to arginine. Prior studies have shown that glucose, arginine, and leucine are effective stimuli of insulin release from the anolian β cell (2, 3, 26). However, the influence of other substrates on α and β cell function is virtually unknown for these animals. Similarly, there is a paucity of information regarding substrate utilization by peripheral tissues in lizards. Nevertheless, some studies provide suggestive evidence of a major role for lipid metabolism in lizards (1, 27–30). Therefore, it seems realistic to consider the possibility that lipids and/or protein may have a preeminent role in saurian homeostasis and that metabolic substrates other than glucose may have a substantial regulatory role in islet cell function in these animals.

Summary. Glucagon secretion from splenic pancreata of *Anolis carolinensis* was not significantly increased by a 56% decrease in the concentration of glucose (1 mg/ml). When the glucose concentration was reduced to 0.5 mg/ml, a significant increase in glucagon release was observed. Arginine (10 m*M*) did not significantly alter glucagon secretion. The pattern of glucagon secretion under stimulatory conditions shows a rapid onset and long duration. A concentration of glucose of 6 mg/ml failed to significantly decrease glucagon release. The results demonstrate the utility of the anolian splenic pancreas for exploring the regulation and dynamics of glucagon secretion.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 184–188 (1978)

Mechanisms of Action of Testosterone on Hemopoiesis *in vivo* and in Diffus Chambers (40017)

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Introduction. It is now well established that androgenic hormones are capable of stimulating erythropoiesis in both experimental animals (1–3) and patients with various types of anemias (4, 5). However, the mechanisms by which these substances influence erythropoiesis remain unclear. It has been reported by several investigators that the erythropoietic effect of androgens is, in part, due to their ability to enhance the endogenous production of erythropoietin (ESF) (1.6–9). In other studies, the direct addition of androgens to *in vitro* marrow cultures has led to augmented erythroid differentiation. Using an *in vitro* methyl cellulose gel system, testosterone produced a significant increase in erythroid colony formation in both rabbit (10, 11) and human bone marrow cultures (12). In addition, in this system, a synergistic effect of a combination of testosterone and erythropoietin on erythroid colony formation was seen. This suggests that, in the presence of testosterone, a greater number of erythropoietin-responsive cells (ERC) is available for ESF to differentiate into the nucleated erythroid cell line.

Clinical experience with androgens in the therapy of aplastic anemia and of neutropenia induced by cytotoxic drugs suggests that these hormones may also stimulate granulopoiesis. The mechanisms by which androgens affect the granulocytic line are still largely unsettled. Rosenblum and Carbone (13) recently showed that *in vitro* addition of testosterone to cultures of human marrow increased the number of granulocyte colonies (CFU-C), and on cytokinetic grounds they attributed this effect to a direct stimulation of CFU-C proliferation.

In addition to these effects on more differentiated hemopoietic cells, testosterone may also have an effect on the pluripotential stem cell compartment. (14, 15) found increasing sensitivity ripotential stem cells or spleen forming units (CFU-S) to the lethal of high specific activity tritiated dine in testosterone-treated polycy mice. CFU-S of normal adult mi generally insensitive to the cytocic tion of [^{3}H]thymidine. Hence the ef testosterone was interpreted as eith gering CFU-S from resting (G_0) sta cell cycle, or shortening the cell c CFU-S.

The present study was undertal examine the granulopoietic effect of terone in mice, using an *in vivo* di chamber (DC) culture system. Al *in vivo* studies are usually complica neutrophil egress from the marrow tight diffusion chambers allow for sessment of total granulocytic diffe tion.

To investigate the possibility of a action of testosterone on the morp cally unrecognizable progenitor cell effect on stem cell populations wa studied in intact mice. Both pluripc spleen colony-forming cells (CFU-S recognizable granulocytic and er cells were evaluated and compared sults in the diffusion chamber studies.

Materials and methods. Femal mice (20–25 g), 10–14 weeks of age used in all experiments.

Long-acting testosterone ena (Tstr) (Testoviron Depot, Shering Cc injected intramuscularly as an en in sesame oil. Both intact animal diffusion chamber-carrying mice re a single injection of 2.5 mg of long testosterone on Day 0. Controls fo intact and chamber-carrying mice injected with the same volume of t hicle, sesame oil.

In vivo studies. At Days 0, 1, 3,

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eatment, a bone marrow cell sus- was obtained from the tibia of o five mice by flushing out the and drawing it back and forth a 24-gauge needle. The total nu- cellularity was determined by a counter Model B. Tibial marrow it least three mice was pooled in Hank's BSS with 100 units of potas- nicillin G and 100 μg of streptomy- ate per milliliter.

suspension was then adjusted to 5 cells/ml and 0.2 ml was injected into ed recipients to assess the number row CFU-S by the spleen colony (16). Bone marrow smears for the ntial counts were made from the ateral tibia.

usion chamber studies. The diffu- amber (DC) technique as described estad (17) was used. On Day 0, bone cells were suspended in Hank's ontaining 10% homologous serum, of potassium penicillin G, and 100 streptomycin sulfate per milliliter. cells were counted in duplicate in a tometer, and the cell suspension ljusted with medium to obtain the concentration. Each chamber was ith 5×10^5 cells/0.1 ml. Two cham- ere implanted surgically into the eal cavity of each recipient mouse light ether anesthesia. Chambers emoved 1–7 days after implantation aken in a 0.5% Pronase and 5% solution in Hank's BSS for 75 min, ells were harvested by washing the er interior three times with Hank's The cell concentration per DC was ined by hemocytometer count. were prepared with a Shandon cy- ifuge and stained with May-Grün- iemsa, and differential counts of at 00 cells were made.

were classified as immature prolif- erythroblasts (proerythroblasts sophilic erythroblasts), mature ery- sts (polychromatophilic and or- matic erythroblasts), proliferative cyte precursors (myeloblasts, pro- ytes, and myelocytes), mature non- ative granulocytes (metamyelo- and granulocytes), macrophages, lymphocytes, and other cells (megakaryo- cytes, unrecognizable, and nonhemo- poietic cells).

The number of CFU-S per DC was deter- mined as follows. Contents from six to eight DCs were pooled and 1×10^5 cells were injected into groups of seven to nine mice previously given 950-R total-body ir- radiation with an X-ray apparatus under the following conditions: 200 kV, 20-mA filtration, 0.5 mm Al + 0.5 mm Cu, 70 rads/min dose rate. Eight days after trans- plantation the animals were killed, the spleens fixed in Bouin's solution, and the spleen colonies counted indepently by two observers.

Results. Intact mice. No differences were found between the bone marrow cell counts of the testosterone-treated mice and the sesame oil control-treated mice. Both control and testosterone-treated animals had total cell counts per tibia ranging between 11.3 and 13.1×10^6 on the first to seventh day of the study.

One day after testosterone treatment a moderate decrease in myeloid cells and a significant increase in erythroid cells were noted. The absolute numbers of myeloid cells were reduced from 4.8×10^6 to 4.0×10^6 per tibia ($P < 0.01$); erythroblasts rose from 3.2×10^6 to 4.0×10^6 cells per tibia ($P < 0.001$). Three days after treatment the total erythroid precursors were still above the control values, 3.7×10^6 as compared to 2.6×10^6 in control animals ($P < 0.01$). By the seventh day, erythroid cells had returned to control values.

The changes in the CFU-S levels of the two groups are shown in Fig. 1. The tibial marrow CFU-S content increased to 166% of control values ($P < 0.05$) on Day 3 after treatment and returned to normal range by the seventh day.

Diffusion chamber studies. Figure 2 shows the total number of nucleated cells harvested from the DCs. In the chambers of testosterone-treated hosts, the growth pattern of bone marrow cells showed an increase in total cell number as compared to the controls. After 3 days of culture the total cell count increased from 3.7×10^5 in controls to 6.1×10^5 in testosterone-treated animals ($P < 0.05$). Differential counts of

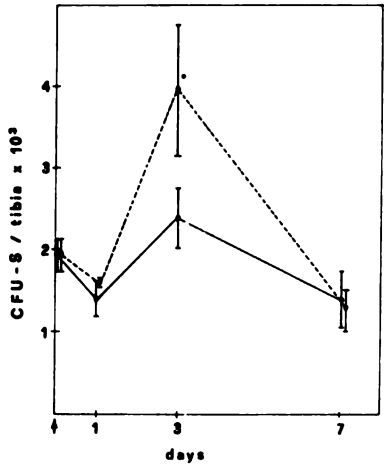

FIG. 1. The total number of CFU-S per tibia of Tstr-treated CF_1 mice (2.5 mg at Day 0) (O-----O) and controls (0.2 ml of sesame oil) (●——●). Values are means ± 1 SE of 7-14 samples from four separate experiments. (*) $P < 0.01$.

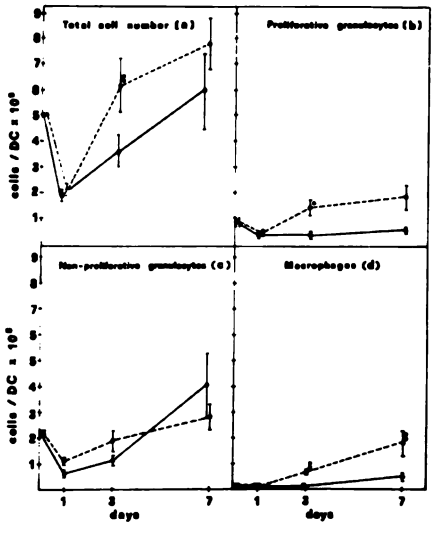

FIG. 2. (a) The total number of nucleated cells harvested from the DCs in Tstr-treated CF_1 host mice (2.5 mg at Day 0) (O-----O) and controls (0.2 ml of sesame oil) (●——●). (b)-(d) Changes with culture time of cell populations harvested from DCs. Values are means ± 1 SE of 6-11 samples from three to six separate experiments. (*) $P < 0.01$; (**) $P < 0.05$.

the cultured cells showed that erythroblasts declined soon after seeding; no difference was observed between control and steroid-treated animals. From the third day of culture onward, no nucleated erythroid cells were observed in the DCs. In DCs of testosterone-treated hosts, the number of proliferative granulocytes on Day 3 was significantly higher ($P < 0.05$) than controls, 1.4×10^5 and 0.46×10^5, respectively. A parallel increase in macrophages was observed on the third and seventh days of culture in the testosterone-treated animals. Lymphoid cells were present, but did not change in number throughout the culture period. Figures 2b, c, and d illustrate the growth curves of the major classes of cells present.

Diffusion chamber CFU-S. The CFU-S content of DCs implanted in testosterone and control-treated host mice is shown in Fig. 3. Although a marked decline in CFU-S was observed, levels of colony-forming cells in testosterone-treated hosts were significantly higher than in control animals on Days 1 and 3 of culture. No difference was observed after 7 days of culture.

Discussion. The numbers of cells harvested from 1-, 3-, and 7-day diffusion cultures showed an increased cellularity in testosterone-treated hosts. These changes were due to proliferation of both granulocytic cells and macrophages. In contrast, the tibial marrow cellularity did

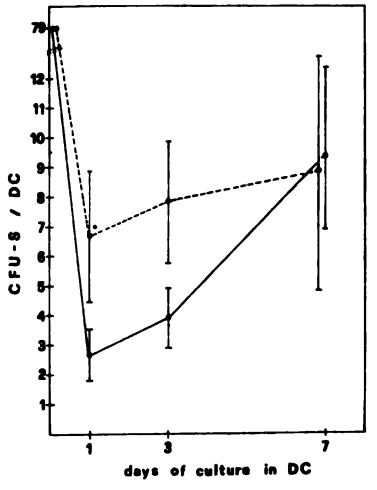

FIG. 3. The total number of CFU-S recovered in DCs implanted in Tstr-treated host mice (2.5 mg at Day 0) (O-----O) and controls (0.2 ml of sesame oil) (●——●). Values are means ± 1 SE of 10-23 DCs from four separate experiments. (*) $P < 0.1$.

ow any significant difference be-
the two treatment groups. This dis-
y observed between the DC and
cellularity can be explained by
t that the DC system allows for
ation and maturation of hemo-
elements, but cellular migration is
ed (17).

n *in vivo* environment, alterations
marrow granulocyte reserve pool
anulocyte migration from the mar-
uld account for changes in total
cellularity. Although no increase
l cells was observed, detailed mar-
fferentials showed an increase in
rcentage of erythroid elements 3
ter treatment. In contrast, the DC
showed a sequential increase in
granulocytic cells and macro-
throughout the culture.

results of *in vivo* CFU-S studies
hat testosterone causes an increase
number of CFU-S in the tibial
3 days after injection. In the DC
injection of testosterone increases
mber of recoverable CFU-S after 1
lays of culture. Since both groups of
showed a marked decline in
r CFU-S, it is uncertain whether
served increase in the testosterone-
l mice was caused by an enhanced
ation rate or decreased cell loss at
el of the undifferentiated compart-
However, these data are in close
ent with those of Byron and Testa
Byron (15), and Fisher *et al.* (12),
owed that testosterone has a rapid
effect which triggers CFU-S into
The slower CFU-S growth phase
in the testosterone-treated group
ne third day of DC culture (Fig. 3)
e related to CFU-S differentiation
nulocytic cells.

es have shown that testosterone
an increase in plasma erythropoi-
tivity (1, 7, 8). Although ESF might
he CFU-S by causing erythroid dif-
ation, several observations suggest
stosterone has a direct effect on
oietic stem cells. Testosterone
increased proliferation of CFU-S in
hemic animals in which there is no
ble ESF. Moreover, the stimulating
effect of testosterone on marrow erythroid progenitor cells is not blocked by the simultaneous administration of anti-ESF serum (19).

The present data are consistent with the view that testosterone may have a direct effect on certain hemopoietic stem cells. In the DC system there is no erythroid differentiation, therefore the effect of testosterone on the CFU-S would not appear to be mediated by ESF. Instead, the enhanced numbers of CFU-S and granulocytic cells would appear to be due to a direct effect on the pluripotential and granulocytic stem cell compartments.

Rosenblum and Carbone (13) recently found increased numbers of colonies following *in vitro* addition of testosterone to cultures of human marrow. They attributed this effect to a direct stimulation of CFU-C proliferation. Our findings would be compatible with such a mode of action and, if this were the case, the observed granulopoietic effect of testosterone may have been due to its action on either the CFU-C or the CFU-S compartment.

In conclusion, we observed an absolute increase in CFU-S both in bone marrow and in diffusion chambers 3 days after injection of testosterone. The enhanced proliferation of the uncommitted progenitor cells is followed by increased bone marrow erythropoiesis and by granulocytic macrophage proliferation in the DC system. It is unclear whether testosterone acts at the CFU-S or at the committed compartment level, and further work is necessary to delineate the effect of this steroid on these stem populations. Our experimental data support the hypothesis that testosterone has more than one action on the hemopoietic system: by enhancing ESF production and by stimulating the stem cell populations. The further differentiation toward the granulocytic or erythroid lineage may be due to perturbations of the homeostatic mechanisms under various types of stress or injury and to the inductive action of the microenvironment as well.

Summary. Injection of long-acting testosterone markedly enhanced bone marrow CFU-S proliferation *in vivo* and CFU-

S recovery in a diffusion chamber (DC) system.

In the DC system the enhanced CFU-S proliferation led to increased cellularity due to an increase in dividing granulocytic elements and macrophages. *In vivo*, no difference was observed in the tibial total nucleated cellularity between treated and control animals. Detailed marrow differential counts, however, showed a significant increase in erythroid cell precursors 1–3 days after testosterone injection.

The relationship between colony-forming units and colony-forming cells is discussed and it is suggested that testosterone may act on different levels of the hemopoietic system. This steroid appears not only to enhance but also to induce proliferation of the uncommitted progenitor cells.

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thione-S-Transferase Activity in the Rabbit Nephron: Segmental Localization solated Tubules and Formation of Thiol Adducts of Ethacrynic Acid (40018)

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ndin is a basic protein isolated from ɔernate of rat and human kidney and ıomogenates (1, 2). In the rat and is the major glutathione (GSH)-S-rase (3). Conjugation with GSH is 'st step in mercapturate formation is the major nonoxidative detoxifica-'stem for many drugs and chemicals ng ethacrynic acid (4). Administra-: ethacrynic acid results in formation ıtathione (EA-GSH) and cysteine yst) adducts of the diuretic (5). The has a significantly greater diuretic on the isolated thick ascending limb ıle's loop than does ethacrynic acid he present studies were designed to e GSH transferase activity in the ɔn and to determine its role in forma-: thiolated adducts.

ıods. Isolation of nephron segments. ; were performed on rabbits since the ɔn segments of this species can be ied and isolated by microdissection Normal female white New Zealand ·, approximately 2 kg in weight, were .ined on a diet of standard rabbit The animals were killed by cervical .tion and the left kidney was immedi-emoved. A 1- to 2-mm-thick trans-ɜection and a 1- to 2-mm tangential ı of superficial cortex were removed ınsferred to a dish of chilled McCoy's l culture medium without phenol red l Island Biological Co., N.Y.), which ·rved as the dissecting medium. The n was bubbled with 95% O_2–5% ndividual tubular segments were sep-by microdissection using a dissecting cope with a calibrated eyepiece reti-oximal convoluted tubules were dis-from the superficial cortical section e other tubular segments from the ·rse section. Proximal convoluted tubules, proximal straight tubules, combined thin descending and ascending limbs of Henle, combined cortical and medullary thick ascending limbs, and combined cortical and medullary collecting tubules were separately isolated. Accuracy of identification was confirmed by measuring the characteristic transtubular potential difference in representative segments using the *in vitro* perfusion technique (7, 8). An approximate total length of 20–40 mm of each segment was pooled in separate test tubes containing approximately 0.2 ml of chilled dissecting medium. Tubular segments were washed twice with 20 ml of medium and centrifuged to remove enzyme liberated during dissection. Aliquots (0.2 ml) of medium were similarly treated and assayed for enzyme activity and did not have GSH transferase activity. Aliquots (0.4 ml) of medium containing the tubular segments were rapidly frozen, thawed, and sonicated for 15 sec in a Sonifier cell disruptor (Heat Systems-Ultrasonic, Inc., Plainview, N.Y.). Total protein was determined using the method of Lowry *et al.* (9). GSH transferase activity was determined on 0.1 ml of supernate representing a total length of 5–10 mm of tubule.

GSH transferase assay. Glutathione transferase activity was determined by monitoring formation of enzyme-catalyzed coupling between glutathione and 1-chloro-2,4-dinitrobenzene (E 9600 at 340 nm) (4).

Aliquots (100 μl) of tubule supernate in bicarbonate buffer (pH 7.4) were added to a 1-cm-path spectrophotometer cell containing 3 ml of a solution of 2.5 mM glutathione and 1.33 mM 1-chloro-2,4-dinitrobenzene in 0.1 M phosphate buffer at pH 7.4. The blank cell contained only reagents. Changes in absorption were followed at 25° in a Pye Unicam 1800 recording spectrophotometer.

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Units of enzyme activity were expressed as micromoles of coupling product formed per minute per microgram of protein.

Formation and liberation of ethacrynic acid adducts by intact tubules. Slices (1 mm) of superficial renal cortex and papillary tip were transferred to a dish of chilled ultrafiltrate of normal rabbit serum prepared by ultrafiltering serum through a Diaflo PM membrane (Amicon Corp., Lexington, Mass.) which effectively excludes molecules of greater than 10,000 MW. The ultrafiltrate was used as the dissecting and incubation medium. (Binding of ethacrynic acid to serum proteins was greater than 99% at 37°.) Teased tubule preparations were prepared from cortical and papillary slices by gentle separation of tubule fragments with fine forceps under a dissecting microscope. The superficial cortical slice was composed almost entirely of proximal tubules, whereas the papilla contained only loops of Henle and collecting tubules.

Two samples of each preparation (10%, w/v) were transferred to individual wells of a microtiter plate (Falcon Plastics) and incubated in ultrafiltrate containing 10^{-4} *M* [^{14}C]oxylacetic-2-ethacrynic acid[1] (sp act, 16.4 μCi/mg) for 30 min at 37° in a humidified atmosphere of 95% O_2–5% CO_2. The protein content of each well was determined by the method of Lowry *et al.* (9). The concentration of EA adducts in the incubation medium did not increase between 15 and 30 min of incubation. Two samples of the isotope-containing ultrafiltrate without tissue were also incubated simultaneously. The incubation medium was collected into capillary tubes and 10 μl was placed on precoated silica gel F-254 thin-layer chromatography plates (Brinkman Instruments, Westbury, N.Y.). Each sample was superimposed upon a spot containing 10% ethacrynic acid and 10% ethacrynic acid–glutathione adduct (prepared in crystalline form and provided by Dr. D. Koechel, Department of Pharmacology and Therapeutics, Medical College of Ohio, Toledo, Ohio). The plates were developed in benzene:methanol:acetic acid (45:8:4). Ethacrynic acid and ethacrynic acid–glutathione spots were localized under uv light (253.7 nm), and individual areas were scraped and transferred to counting vials containing 10 ml of Aquasol liquid scintillation fluid (New England Nuclear) for radioactive counting.

The area between the ethacrynic acid and ethacrynic acid–glutathione spots contained ethacrynic acid adducts of intermediate molecular weight. This region was removed and counted separately. Two 10-μl samples of the incubation medium from each well were pipetted directly into counting vials. Samples were counted in a Packard–Tricarb liquid scintillation spectrophotometer with correction for quenching. Total recovery of isotope from the plate was expressed as a percentage of counts obtained by direct counting of an equal volume of the same sample. Absolute values were obtained from the thin-layer plates for this recovery factor which exceeded 80% in all experiments. Ninety-six percent of [^{14}C]ethacrynic acid radioactivity was recovered from the ethacrynic acid spot, 2.5% from the ethacrynic acid–glutathione spot, and 1.5% from the intermediate zone.

In order to determine the proportion of ethacrynic acid–cysteine adduct (EA–Cys) in the "unidentified adducts" obtained from the thin-layer plates, three additional experiments were performed on proximal tubule suspensions in which separation of the ethacrynic adducts was achieved by paper chromatography as described by Duggan and Noll (10). An EA–Cys marker was prepared by dissolving equimolar amounts of ethacrynic acid and L-cysteine in water. Because free cysteine was present, the samples were applied to the paper adjacent to the EA, EA–GSH, and EA–Cys markers to prevent formation of EA–Cys by its combination with free ethacrynic acid in the sample. The area between the EA and the EA–GSH zones was removed after development and divided into an EA–Cys section and a remaining section containing other unidentified adducts. Radioactivity was counted using Toluene Puresolve liquid scintillation cocktail (Packard Instruments Co., Downers Grove, Ill.).

Tissue content of ethacrynic acid adducts. Following incubation, proximal tubules

[1] Supplied by Dr. Frank Wolf, Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

ɔtted dry, rapidly washed in incuba-
:dium, transferred to small tubes
ng 100 μl of benzene:methanol:
ıcid (45:8:4), disrupted by sonica-
ıd extracted for 1 hr. The extracts
parated on thin-layer plates as de-
above.

*ts. Localization of glutathione trans-
ctivity*. Enzyme activity was confined
proximal convoluted and proximal
tubules and was not detectable in
n descending and ascending loops,
scending limbs, or cortical and med-
ollecting tubules.

/ity (μmoles of conjugate/μg of pro-
n) was 0.23 ± 0.04 in the convoluted
ı and 0.25 ± 0.09 in the straight
ı of the proximal tubules.[2] Duplicate
ements on the same sample gave
al results.

*ıation of ethacrynic acid adducts by
ubules. a) Spontaneous formation in
ion medium*. Figure 1 illustrates the
ıt following incubation of an ultrafil-
f rabbit serum with [^{14}C]ethacrynic
0^{-4} *M*) in the absence of renal tissue,
:tivity recoverable as ethacrynic acid
luced to 7.4 ± 1.1 × 10^{-5} *M* concom-
ith the appearance of EA- GSH (0.5
× 10^{-5} *M*) and other EA adducts
0.1 × 10^{-5} *M*).

Formation of adducts by renal tubules.
experiments, both proximal tubules
enle's loops plus collecting tubules
emoved from the same kidney. The
ɔrotein content (±SE) per incubation
proximal tubules was 291 ± 22 μg,
ıat of Henle's loops plus collecting
s was 262 ± 36 μg.

oth groups of tubules the concentra-
f ethacrynic acid in the incubation
m fell significantly and the levels of
SH increased. The increment ob-
with proximal tubules was signifi-
greater than that with papillary seg-
($P < 0.05$). Since GSH transferase
was not detected in the latter neph-
gments, it is assumed that this repre-
noncatalytically formed EA-GSH
is liberated into the incubation me-

ults are the means ± SE of enzyme assays on
ıals.

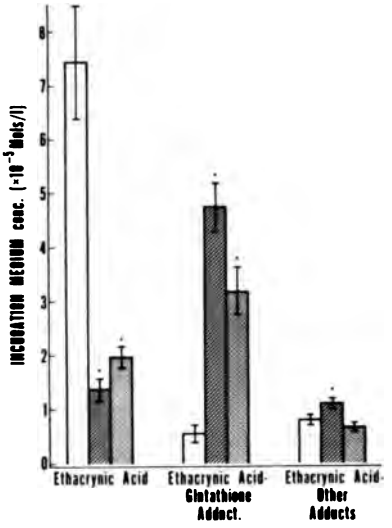

FIG. 1. Medium concentrations of ethacrynic acid, ethacrynic acid–glutathione, and other ethacrynic acid adducts following incubation of rabbit serum ultrafiltrate with [^{14}C]ethacrynic acid in the absence and presence of renal tubules. Open bars = no renal tissue; cross-hatched bars = proximal tubules; stippled bars = Henle's loops and collecting tubules. (*) $P < 0.05$ vs medium with no tissue.

dium. No additional ethacrynic acid adducts were formed by the loops and collecting tubules, whereas there was a significant increase in the medium concentration of other "unidentified adducts" following incubation of proximal tubules (Fig. 1). The difference between the original medium concentration of isotope and total isotope recovery following incubation represents the amount removed by nonspecific tissue binding.

Paper chromatography of the unidentified adducts (as defined by thin-layer chromatography) formed by proximal tubules revealed that 61.3 ± 9.2% of the radioactivity was recoverable as the ethacrynic acid–cysteine adduct.

(*c*) *Tissue content of ethacrynic acid and its adducts*. Analysis of the relative distribution of adducts within the proximal tubular cells revealed 15.0 ± 4.6% as ethacrynic acid, 21.3 ± 4.1% as unidentified adducts, and 63.8 ± 3.5% as ethacrynic acid–glutathione.

Discussion. Cellular localization of enzyme activity in different parts of the neph-

ron has hitherto been based on histochemical techniques using freeze-dried sections of tissue (11). More recently a method of enzyme localization has been utilized in which isolated viable segments of the rabbit nephron are removed and assayed for enzyme activity *in vitro*. Imbert and co-workers (12) and ourselves (13) have measured adenylate cyclase activity in segments of the rabbit nephron. The present study represents an additional application of this technique. The glutathione transferase assay was sufficiently sensitive to detect activity in proximal tubular segments 2–3 mm in length. In order to ensure that false negative results were not obtained on segments with relatively low protein content per unit length, e.g., the thick ascending limb (12), a minimum of 5- to 10-mm length of each segment type (containing at least 0.3 μg of protein) was used.

The results indicate that GSH transferase activity in the rabbit nephron resides entirely in the proximal convoluted and proximal straight tubules. Studies in rats demonstrate the identity of glutathione transferase B with ligandin (3) and its localization by immunofluorescence to the proximal tubules of the renal cortex (14). Selective poisoning of the proximal tubule of the rat kidney by heavy metals leads to the appearance of ligandin in the urine and suggests that it is localized preferentially in the terminal parts of the proximal tubule in this species (15). Ligandin has not been purifed from rabbit kidney and anti-rat ligandin IgG does not immunologically cross-react with rabbit tissues. In rats, ligandin is the predominant GSH transferase (16). It is not known whether rabbit kidney contains more than one GSH transferase which catalytically reacts with 1-chloro-2,4-dinitrobenzene.

The evidence that GSH transferase plays a role in organic anion transport by the proximal tubules is inferential and includes studies of competitive binding of ligands, such as penicillin, probenecid, and Bromsulphalein, to renal ligandin, and an increase in renal organic anion excretion following induction of ligandin (2). Since organic anion transport by renal tubular cells involves active transport at the level of peritubular or luminal membranes, failure to demonstrate ligandin in plasma membrane fractions of kidney suggests that binding of ligands to GSH is not directly coupled to the active transport processes. Since conjugation to GSH is the first step in the mercapturic acid pathway, GSH transferase may result in formation of GSH adducts.

In the present study, separated renal tubules were incubated *in vitro*. These tubules retain viability for several hours and demonstrate transport activity (17). Since the tubules are fragmented with their lumens communicating with the external medium, substances transported actively or diffusing across both luminal and peritubular surfaces appear in the incubation medium. In the present study intact proximal tubules, which have glutathione transferase activity, form EA-GSH and EA-cysteine which enter the tubular fluid and presumably act on more distal nephron sites. Papillary nephron segments are deficient in this enzyme activity and form significantly less glutathione or other adducts.

Summary. Ligandin is a major renal organic anion-binding protein and is the major glutathione (GSH)-S-transferase in the rat, monkey, and man. GSH transferase activity was measured in isolated segments of the normal rabbit nephron using 1-chloro-2,4-dinitrobenzene as substrate. Enzyme activity was confined to the proximal convoluted and proximal straight tubules and was not detectable in the loops of Henle and collecting tubules.

Incubation of proximal tubules with [^{14}C]ethacrynic acid resulted in formation of glutathione and cysteine adducts. Loops of Henle and collecting tubules formed significantly less glutathione adduct and no other adducts. GSH transferase in the proximal tubule may thus be important *in vivo* in the formation of thiolated adducts by the kidney.

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Methylation of the Lysine Residues of Monellin[1] (40019)

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Monellin, a sweet-tasting protein, has been isolated in homogeneous form and characterized (1–10). Prior to the present study, a number of unsuccessful attempts were made in our laboratory to derivatize monellin without destroying its sweet taste. In this paper we describe methylation of the lysyl residues, a portion of which can be so modified with retention of the sweetness of the protein. Through this reaction a radioactively labeled derivative of monellin has been prepared, which will now enable direct binding studies of it to taste receptors.

Materials and methods. Monellin was isolated from the fruit of *Dioscoreophyllum cumminsii* (1, 4). Reagents and their sources were as follows: fluorescamine, Roche Laboratories; 37% formaldehyde, Fisher Scientific; [^{3}H]formaldehyde (0.1 Ci/mmole, lot No. 728-029), New England Nuclear Corp.; N^{ϵ}-trimethyl-L-lysine-bis(*p*-hydroxyazobenzene-*p'*-sulfonate) $2H_2O$ (A Grade), Calbiochem; N^{ϵ}-methyl-L-lysine HCl and N^{ϵ}-dimethyl-L-lysine HCl, Cyclo Chemical. Other chemicals were reagent grade.

Methylation of monellin (11). The protein was dissolved in 0.2 *M* sodium borate, pH 9.0, at 10 mg/ml (1 m*M* monellin, or 11 m*M* in primary amino groups). Concentrated $NaBH_4$ in water (10 mg/ml) was added to give a final concentration of 0.5 mg/ml, which is an approximate equimolarity of amino groups and borohydride. The mixture was kept at 0°. Small alic formaldehyde (1–5 μl of 6–8 *M* fo hyde/ml of monellin solution) wer at 5-min intervals with stirring. Just each addition, an aliquot of the was removed for chemical determin the extent of reaction and for taste The extent of methylation was meas assaying the free amino groups re (12).

Tritium-labeled methylated mone prepared with [^{3}H]formaldehyde μmole). The unreacted formaldeh removed with an Amicon ultrafiltra paratus (Model 202, UM2 filter) temperature. The monellin solution tered and then alternately diluted (w *M* sodium phosphate buffer, pH 7 concentrated until the radioactivity effluent was reduced to backgroun The ^{3}H-labeled methylated mone chromatographed on a column of lulose. The fractions were assayed tein concentration, for free amino and for radioactivity by liquid scin counting (13) with a Packard Model 3375 at a counting effici 37%.

Amino acid analyses. Samples of r were hydrolyzed *in vacuo* in constan HCl for 22 hr at 110°. A Beckm automatic analyzer equipped with man System AA computing integra used. The standard basic columr Beckman PA-35 resin) was eluted w *N* sodium citrate buffer, pH 5.28, at a flow rate of 70 ml/hr. On this monomethyllysine and dimethyllysi not separated. For their separatio cm column of Durrum DC-6A re used. It was eluted initially with sodium citrate buffer, pH 5.70, at 70 min the temperature was raise and the buffer changed to 0.35 *N*

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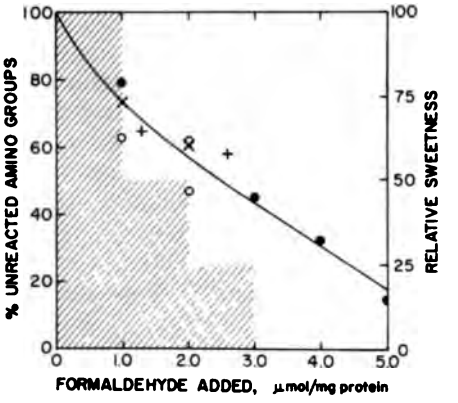

FIG. 1. Methylation of monellin. Aliquots of formaldehyde were added as shown on the abscissa. The ordinate shows sweetness level (shaded area) and fluorescamine reactivity remaining in four experiments (○, x, ●, +). The sweetness of the samples was evaluated at three concentrations (5, 10, and 20 μg/ml); the lowest concentration that tasted sweet is indicated on the scale as 100 (i.e., 100 means sweet at 5 μg/ml, while 0 means not perceptibly sweet at 20 μg/ml).

ɛ, pH 4.70. The flow rate was 70 ml/ roughout. Elution times of the stan- to the nearest minute were as follows: , 67; monomethyllysine, 76; dimethyl- , 81; trimethyllysine, 84; histidine, nmonia, 100; and arginine, 195.

' electrophoresis. Electrophoresis was d out in polyacrylamide gels under al conditions: pH 6.9 (0.05 *M* ammo- acetate), pH 7.5 (0.05 *M* ammonium te + 8 *M* urea + 0.1% 2-mercaptoeth- , pH 8.4 (0.05 *M* Tris/acetate), and 0.0 (0.05 *M* sodium glycinate). In gels th 7.5 and 10% (w/w) acrylamide the of acrylamide to *N,N'*-methylenebis- mide was 30:1. Electrolyte buffers identical with those in the gels except one of them contained urea. Proteins stained for 1 to 2 hr in a solution of black (1%) and Coomassie brilliant G250 (0.25%) in 7% acetic acid, or atively with Coomassie brilliant blue (0.25%) alone (14). Gels were ed at 560 or 600 nm.

two chains of monellin were disso- l by incubation for 2 hr at 25° in 8 *M* 0.01 *M* Tris/acetate buffer, pH 8.4, % mercaptoethanol (final pH, 8.5). ation was achieved by polyacrylamide ectrophoresis in the presence of 8 *M* + 0.1% mercaptoethanol.

umn chromatography and other pro- es. Chromatography on CM-cellulose arried out in 0.01 *M* sodium phosphate r, pH 7.2, using elution with a linear ent of 0 to 0.05 *M* NaCl in the buffer el filtration was carried out on Seph- G-75 in 0.05 *M* Tris/acetate buffer, .4, which contained 0.2 *M* KCl to nt adsorption of the protein. Protein escence (2, 9) and sweetness (1) were mined as described earlier. Protein ntrations were calculated by compari- vith the fluorescence emission of a ard solution of native monellin.

ults and discussion. Methylation and taste. Monellin that was successively ylated showed successive decreases in ry amino groups (Fig. 1), 20 to 40% ich could be methylated without ap- ble loss of sweetness. Additional ylation resulted in decreased sweet- Even after extensive methylation un- enaturing conditions (2% sodium do- decyl sulfate or pH 11.5), about 20% of the amino groups remained fluorescamine reactive.

Chromatography of methylated monellin. In order to determine the possible extent of heterodispersity, the CM-cellulose chromatogram was analyzed for protein fluorescence, primary amino groups, and sweet taste. The elution profile (Fig. 2) showed a single peak for which these parameters were coincident. The ratio of the fluorescence parameters in the more slowly eluting material indicated somewhat less extensive methylation. A mixture of methylated and unmethylated monellin gave essentially the same elution profile, although it appeared to be slightly broader.

The half-width of the fluorescence emission for the fractions in the midportion of the elution peak (Fig. 2) was relatively constant at 62 ± 2 nm (range) (native monellin is 60 nm) (9). The fractions beyond number 215 showed an emission peak at 304 nm due to tyrosine. The data therefore indicate some conformational alteration of the protein due to methylation.

Amino acid composition of methylated monellin. The amino acid compositions of the six pooled fractions (see Fig. 2) were very similar (Table I), and those of pools

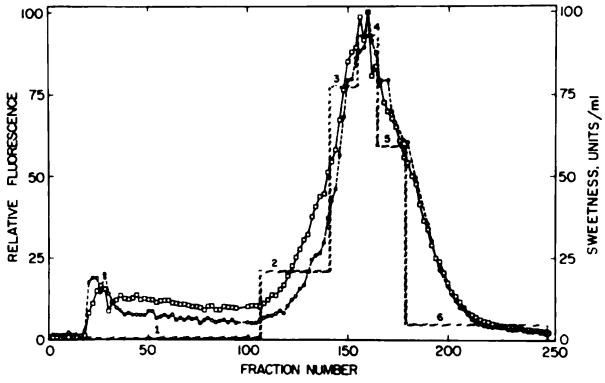

FIG. 2. Chromatography of methylated monellin on CM-cellulose. Methylated monellin (40 mg) (approximately 50% methylation) was chromatographed on a 2 × 20-cm column of CM-cellulose. The sample was applied in 0.01 *M* sodium phosphate buffer, pH 7.2, the column washed with 40 ml (1 column volume) of the same buffer, and the protein eluted with a 300-ml linear gradient of 0 to 0.05 *M* NaCl in the phosphate buffer. Fractions of 1 ml were collected at a flow rate of 15 ml/hr. Protein fluorescence (protein concentration) (□——□) and fluorescamine fluorescence (primary amino group concentration) (O- - -O) are expressed as relative fluorescence; curves normalized to the peak fraction as 100. The hatched lines are the sweetness values, with the numbers there indicating the six pools. The data for methylated lysines in these six pools are shown in Table II.

TABLE 1. AMINO ACID COMPOSITIONS OF CHROMATOGRAPHIC FRACTIONS OF METHYLATED MONELLIN.

Residue	Pool number[a] (mole%) 1	2	3	4	5	6	Combined pools No. 3 to 6 (mole/mole of protein)[b]	Lit. ref. 2	Lit. ref. 8
Lys (total)	9.3	9.6	9.6	9.6	9.6	9.7	8.22 ± 0.14	8	9
His[c]	Trace	0.3	0.2	Trace	Trace	Trace	0.03 ± 0.07	0	–
Arg	6.1	6.2	6.9	7.0	6.9	6.6	5.85 ± 0.13	7	6
Asx	11.6	10.4	11.1	11.2	11.2	11.3	9.58 ± 0.14	10	10
Thr	4.4	4.7	4.5	4.5	4.3	4.3	3.77 ± 0.04	4	4
Ser	3.3	5.2	3.2	2.8	2.6	3.7	2.79 ± 0.57	2	2
Glx	15.0	14.4	14.2	14.2	13.8	14.0	12.00	12	12
Pro	6.1	5.5	6.0	6.1	6.5	6.3	5.32 ± 0.27	6	5
Cys/2	0.4	0.0	0.5	0.7	0.4	0.4	0.43 ± 0.12	1	1
Gly	10.9	11.2	10.1	10.0	9.8	10.4	8.59 ± 0.19	8	8
Ala	3.0	4.5	3.5	3.3	3.4	3.6	2.95 ± 0.12	3	3
Val	3.6	3.3	3.7	3.6	4.1	3.9	3.17 ± 0.11[d]	4	4
Met	0.8	0.8	0.8	0.9	1.0	0.8	0.75 ± 0.07	1	1
Ile	8.2	6.7	7.1	7.1	7.3	7.1	6.12 ± 0.13[d]	6	8
Leu	5.9	6.0	6.4	6.5	6.4	6.2	5.46 ± 0.08[d]	6	6
Tyr	6.2	6.3	7.0	7.2	7.2	6.4	5.95 ± 0.34[d]	7	7
Phe	5.2	4.9	5.2	5.3	5.5	5.3	4.55 ± 0.15	5	5
Trp	–	–	–	–	–	–	– –	1	1

[a] Pools from the chromatography column shown in Fig. 2. Average values from duplicate analyses.
[b] Mean ± SD.; values normalized to Glx at 12.00.
[c] The presence of His reveals a trace contamination.
[d] Low recoveries of Val, Ile, Leu, and Tyr are probably due to the presence of Ile-Ile, Val-Ile, Ile-Tyr, Val-Tyr, Leu-Tyr, and Tyr-Val bonds in the protein (7, 8).

No. 3 to 6 were identical within experimental error (cf. 2, 8). This demonstrated that the two chains of monellin had not separated. The two chains would not have eluted together on this column and the sweet taste activity would have been lost.

Total methyllysine among the protein pools was similar (Table II). The lower methyllysine content in the trailing edge (pool No. 6) agrees with the fluorescence data (see above) and is accounted for by a decrease only in the dimethyl derivative.

I. EXTENT OF METHYLATION OF LYSINES IN METHYLATED MONELLIN.

Percentage of total lysine[a]			
Methyl-lysine[c]	Methyllysine[d]	Monomethyllysine[d]	Dimethyllysine[d]
48.5	49.6	10.3	39.3
54.0	61.3	6.4	54.8
51.7	53.6	8.2	45.4
47.5	48.0	7.0	41.0
45.4	46.2	8.2	38.0
36.0	38.0	8.8	29.3

es of duplicate analyses. Trimethyllysine ent.

om the chromatography column shown in

resin, 8-cm column.
resin, 20-cm column.

ant percentage of monomethyllyols No. 3 to 6 and the progressive in dimethyllysine in these pools inst the possibility of admixtures ated and unmethylated monellin. rmation of mainly dimethyllysine lin is similar to the results with oteins; reductive methylation of psin, chymotrypsinogen, transfer-yme, and ovomucoid with formalads to formation of dimethyllysine ncipal product (11).

eled methylated monellin. A sam--labeled methylated monellin (27 chromatographed on a column of lose (1.3 × 30 cm). It eluted in a ak similar to that in Fig. 2, with activity, protein fluorescence, and nine fluorescence coincident across n peak. The specific radioactivity uCi/μmole (±10%) in the major f the peak, and for a second prepnder similar, but not identical, s the specific radioactivity was 14 le.

ure of ^{3}H-labeled methylated mo- μg) and unmethylated monellin was chromatographed on a Sephacolumn (1.5 × 41 cm). The small of the ^{3}H-labeled derivative did ere with the protein fluorescence native monellin, enabling detece two molecular species by indeneasures. The two elution peaks efore separately observable. Each geneous with respect to molecular size. The methylated monellin eluted somewhat earlier than the native monellin, indicating a slightly larger Stokes radius.

Polyacrylamide gel electrophoresis of native and methylated monellins. The six pools of methylated monellin (Fig. 2) and an additional preparation (66% methylated, which tasted sweet) were compared with the native protein by electrophoresis toward the cathode at pH 8.4 on 7.5% gels. The mobility of the methylated protein was in every case less than that of the native, thereby demonstrating the absence of unmodified protein. The mobility decreased from pools No. 6 to 1, consistent with decreasing net positive surface charge. Thus the orders of electrophoretic mobilities and affinities for the cation exchanger were in complete agreement.

Both the native and 66% methylated proteins migrated toward the anode at pH 10.0 (Fig. 3, left), with the methylated protein showing higher mobility. At pH 6.9 (Fig. 3, right) the converse order of mobilities was observed. Because all lysyl residues, whether methylated or unmodified, are fully charged at pH 6.9, this result can be explained either by deamidation or by a methylation-induced conformational change, resulting possibly in increased anion binding. Aggregation could not explain the findings.

Conformational changes that affect electrophoretic mobility should be abolished under denaturing conditions whereas changes in the net charge of the protein should not. Accordingly, native and methylated proteins were denatured and examined by electrophoresis. Monellin was dissociated into its two chains (Fig. 4), one bearing a net positive charge at pH 7.5 (the smaller subunit) and the other a net negative charge (the larger subunit). Methylation had no significant effect on the electrophoretic mobility under denaturing conditions. Thus the change in electrophoretic mobility of the intact protein after methylation must be related to a change in its conformation rather than to deamidation.

The evidence to date indicates that the tertiary structure of monellin is important for its sweet taste activity (1–10). The present work shows that a portion of the lysyl residues can be methylated without appreciable loss of sweet taste activity; further

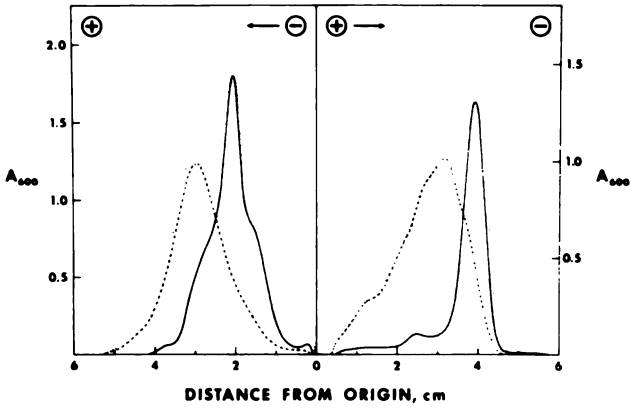

FIG. 3. Left: Electrophoresis in parallel of 50 μg of native monellin (——), 50 μg of 66% methylated monellin (——), and a mixture (50 + 50 μg) of the two samples (· · · ·) toward the anode in 10% (w/w) polyacrylamide gels at pH 10.0 (6 hr, 3 mA/gel). Stained with Coomassie brilliant blue G250. Right: Electrophoresis in parallel of 25 μg of native monellin (——), 50 μg of 66% methylated monellin (——), and a mixture (25 + 50 μg) of the two (· · · ·) toward the cathode in 7.5% (w/w) polyacrylamide gels at pH 6.9 (10 hr, 5 mA/gel). Stained with an amido black-Coomassie brilliant blue G250 mixture.

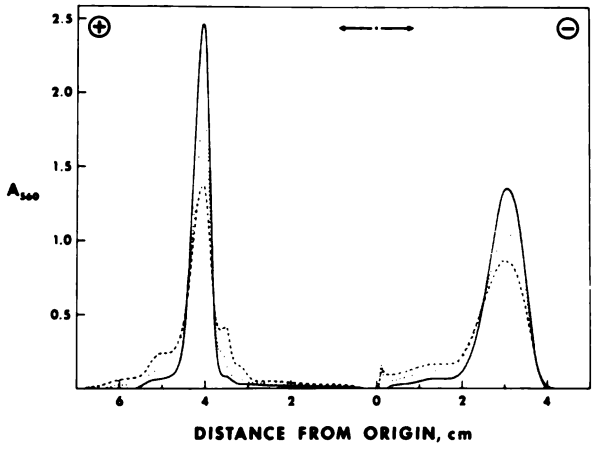

FIG. 4. Electrophoresis in parallel of 50 μg of native monellin (——), 46 μg of 66% methylated monellin (——), and a mixture (25 + 23 μg) of the two (· · · ·) in 7.5% (w/w) polyacrylamide gels containing 8 *M* urea + 0.1% mercaptoethanol, pH 7.5 (24 hr, 2.5 mA/gel). The two monellin chains were dissociated by incubation for 2 hr at 25° in 8 *M* urea + 1% mercaptoethanol, pH 8.5, prior to electrophoresis. Stained as in Fig. 3, right.

methylation results in a decline in sweetness. Methylated monellin shows evidence of a slight increase in Stokes radius, a change in electrophoretic mobility, and a small increase in fluorescence emission half-width, all of which are consistent with a conformation change that includes some expansion of the molecule.

Summary. The ϵ-amino groups of the lysyl residues of monellin were reductively methylated with formaldehyde and sodium borohydride; 20–40% of the lysines could be methylated with essentially complete retention of the sweetness of the protein. The methylated protein yielded dimethyllysine and monomethyllysine upon acid hydrolysis. ^{3}H-Labeled methylated monellin was also prepared with [^{3}H]formaldehyde as the methyl donor; this derivative could be useful in binding studies to taste receptors. The methylated monellin was studied by ion-exchange chromatography, gel filtration, fluorescence spectroscopy, polyacrylamide gel electrophoresis, and amino acid analysis. Although sweetness was maintained after limited methylation, some

change in conformation of the protein did occur.

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Dual Excitatory Effect of Acetylcholine in the Mouse Vas Deferens[1] (4002

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Sympathomimetic effects of acetylcholine and other nicotinic agonists have been described in a variety of tissues (1). For instance, Daly and Scott (2) showed that close arterial injection of acetylcholine into the dog spleen caused contraction which was greatly reduced or abolished by hexamethonium, and by phenoxybenzamine, sympathetic denervation, or pretreatment with reserpine. Similar observations were made in cat and dog heart preparations by Lee and Shideman (3). Subsequent biochemical studies in cat and rabbit hearts provided direct evidence for release of norepinephrine by acetylcholine and other nicotinic agonists (4, 5). The absence of ganglion cells in many of the tissues studied (2, 3) suggested that the nicotinic receptors mediating the release of norepinephrine were located at adrenergic nerve terminals, a view supported by the failure of tetrodotoxin to prevent acetylcholine-induced release from cat spleen (6) or nicotine-induced release from guinea pig hearts (7). In the present study we have examined whether release of norepinephrine may contribute to the contractile response of the mouse vas deferens to acetylcholine. The experiments were prompted by a preliminary observation that the concentration-response curve of acetylcholine in this tissue was biphasic.

Materials and methods. Mice, weighing 30–40 g, were killed by a blow to the head. Vasa deferentia were removed and suspended in organ baths containing 25 ml of modified Krebs' solution of the following composition (millimolar): NaCl, 113; KCl, 4.8; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; $NaHCO_3$, 25; and dextrose, 5.5. The solution was maintained at 37°C and continuously bubbled with 95% O_2:5% CO_2. Contractions of the smooth muscle were recorded isometrically Grass force-displacement transducer (and model 5D polygraph. An initial of 0.5 g was applied and the tissue equilibrated for 1 hr before drug applied to the bath. Concentrat sponse curves were obtained by adc creasing doses of acetylcholine at intervals. Acetylcholine was remo washing when the contractile respor reached its peak. When the effects o methonium, atropine, tetrodotoxi phentolamine on the responses to ace line were examined, these drugs w plied for 1 hr prior to, and throughc period of exposure to acetylcholine.

Results and discussion. Concentrat sponse curves of acetylcholine are sh Fig. 1. In untreated tissues, the me centration–response curve of acetyl was biphasic. Treatment with either carinic antagonist (atropine) or a n antagonist (hexamethonium) separa atypical curve into two simple con tion–response curves. The muscari fects of acetylcholine occurred in th of concentrations from 10^{-6} to 1(while nicotinic effects were restric higher concentrations, approximatel to 3×10^{-3} *M*. The muscarinic re are presumably located on the smoot cle cells. The nicotinic effects we diated by release of norepinephrin they were antagonized by phentol suggesting that the nicotinic recept located on adrenergic neurons. The vation that tetrodotoxin did not the nicotinic effects suggests that potentials are not involved in the rel norepinephrine by acetylcholine in t sue. In other experiments responses mouse vas deferens to transmural el stimulation were inhibited by tetrod Thus the results are consistent wi location of excitatory nicotinic recep adrenergic nerve terminals. Similar sions were reached from studies in c

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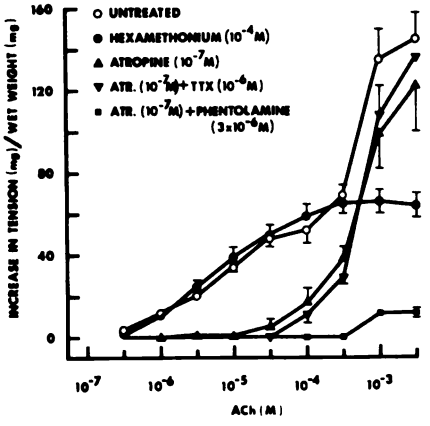

FIG. 1. Mean concentration-response curves of acetylcholine in the mouse vas deferens. Vertical bars represent standard errors. The number of observations at each point ranged from 4 to 12.

guinea pig hearts (5, 7), cat spleen (6), and rat seminal vesicle (8).

The control concentration-response curve in Fig. 1 differs from that obtained in the mouse vas deferens by Jones and Spriggs (9), who found that responses to acetylcholine were linear in the range of concentrations from 10^{-6} to 10^{-3} *M*. Furthermore, the mean maximum tension developed (0.48 g) was considerably less than we observed in controls (1.28 g), but similar to that in tissues treated with hexamethonium (0.66 g). It is possible that the relatively short time intervals between doses of acetylcholine (3 min) used by Jones and Spriggs resulted in attenuation of nicotinic responses due to autoinhibition as shown in the rabbit heart by Löffelholz (10).

Summary. Relatively low concentrations of acetylcholine ($< 10^{-4}$ *M*) contract the isolated mouse vas deferens by interaction with muscarinic receptors. Contractile responses to higher concentrations ($> 10^{-4}$ *M*) may be due to release of norepinephrine following interaction of acetylcholine with nicotinic receptors on adrenergic nerve terminals.

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Effects of Fenfluramine and Norfenfluramine on Brain Serotonin Metabolism in Rats (40021)

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Fenfluramine [(*m*-trifluoromethyl)-*N*-ethyl-amphetamine] is an anorectic drug that depletes brain serotonin in rats (1). A major metabolite of fenfluramine is the *N*-dealkylated product, norfenfluramine (2). Norfenfluramine is structurally similar to *m*-chloroamphetamine and *p*-chloroamphetamine, halogenated amphetamines with well-characterized actions on brain serotonin neurons. We did the experiments described here to characterize further some of the effects of fenfluramine and norfenfluramine on parameters of serotonin neuronal function previously measured with *p*-chloroamphetamine.

Methods. Male Wistar rats (Harlan Industries, Cumberland, Ind.) weighing 130–150 g were given ip injections of fenfluramine hydrochloride or norfenfluramine hydrochloride (A. H. Robins), probenecid (Merck), or fluoxetine hydrochloride (Lilly). The rats were killed by decapitation, and whole brains were rapidly removed, frozen on dry ice, and stored at −15° prior to analysis of serotonin and 5-hydroxyindoleacetic acid (5-HIAA) (3) or tryptophan hydroxylase (4). Monoamine oxidase in brain mitochondria was determined radiometrically with 100 μM [^{14}C]serotonin (New England Nuclear) as substrate (5).

Results. Acute effects. Within 30 min, both fenfluramine and norfenfluramine caused a significant depletion of brain serotonin, the levels of which continued to decline throughout the 4-hr period of study (Table I). Though norfenfluramine depleted serotonin more than did fenfluramine, the differences between the two drugs were small. Brain 5-HIAA levels did not change significantly at 30 min or at 1 hr, but were decreased at 2 and 4 hr.

Since monoamine oxidase inhibition by *p*-chloroamphetamine may contribute to its lowering of 5-HIAA in brain (5), we compared the effects of these compounds as inhibitors of monoamine oxidase *in vitro* (Fig. 1). Fenfluramine and norfenfluramine were virtually identical in their ability to inhibit monoamine oxidase and were less than one-tenth as active as *p*-chloroamphetamine.

The lowering of brain serotonin by *p*-chloroamphetamine is probably largely attributable to a decrease in tryptophan hydroxylase (6). Table II shows that both fenfluramine and norfenfluramine decreased tryptophan hydroxylase activity as well as serotonin concentration acutely in rat brain.

Long-term effects. Table III shows that serotonin concentration in brain was decreased significantly still at 1 week after the administration of fenfluramine or norfenfluramine. Serotonin concentrations were not changed by fenfluramine and norfenfluramine in rats pretreated with fluoxetine an inhibitor of uptake into serotonin neurons.

The turnover of serotonin, measured by the rate of accumulation of 5-HIAA after probenecid injection, was significantly decreased in rat brain 1 week after fenfluramine treatment (Fig. 2).

Discussion. Our findings with fenfluramine and norfenfluramine generally agree with previously published data in areas of overlap and extend the observations that have been made with these two serotonin-depleting drugs. Our results (Table I) showed a slightly greater and more rapid depletion of serotonin by norfenfluramine than by fenfluramine, in agreement with data of Duhault *et al.* (2). Norfenfluramine may contribute substantially to the effects observed after fenfluramine injection, especially at longer times (2).

We have previously suggested that *p*-chloramphetamine exerts multiple actions on brain serotonin neurons—inhibition of tryptophan hydroxylation, inhibition of ser-

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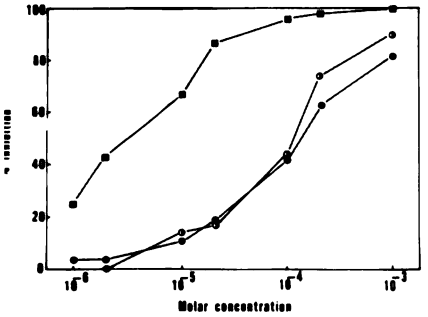
TABLE I. INITIAL EFFECTS OF FENFLURAMINE AND NORFENFLURAMINE ON BRAIN SEROTONIN AND 5-HIAA IN RATS.[a]

Treatment	Time (hr)	Brain 5-hydroxyindoles (μg/g)	
		Serotonin	5-HIAA
Control	–	0.55 ± 0.02	0.56 ± 0.02
Fenfluramine	0.5	0.47 ± 0.02*	0.54 ± 0.02
	1	0.37 ± 0.02*	0.59 ± 0.02
	2	0.30 ± 0.01*	0.47 ± 0.01*
	4	0.24 ± 0.01*	0.38 ± 0.01*
Norfenfluramine	0.5	0.44 ± 0.02*	0.58 ± 0.01
	1	0.31 ± 0.02*	0.52 ± 0.02
	2	0.21 ± 0.005*	0.47 ± 0.01*
	4	0.19 ± 0.01*	0.37 ± 0.01*

[a] enfluramine hydrochloride and norfenfluramine hydrochloride were injected at 15 mg/kg. Mean values ± ard errors for five rats per group are shown.
* ignificantly different from control group, $P < 0.05$.



G. 1. *In vitro* inhibition of monoamine oxidase nfluramine (●), norfenfluramine (◑), and *p*-chlo-phetamine (■). All determinations were made in ate.

TABLE II. EFFECT OF FENFLURAMINE AND RFENFLURAMINE ON SEROTONIN CONCENTRATION D TRYPTOPHAN HYDROXYLASE ACTIVITY IN RAT BRAIN.[a]

atment group	Serotonin (μg/g)	Tryptophan hydroxylase (μg/g/hr)
ne	0.63 ± 0.01	24.6 ± 0.5
fluramine	0.27 ± 0.02*	16.2 ± 0.2*
fenfluramine	0.22 ± 0.01*	17.3 ± 0.7*

Fenfluramine hydrochloride and norfenfluramine chloride were injected at 15 mg/kg 6 hr before s of five rats were killed.
Different from saline group, $P < 0.001$.

in uptake, release of serotonin, and ition of monoamine oxidase (7). Fen-mine may also exert multiple actions, inhibition of monoamine oxidase is ably of no importance in fenfluramine's n based on the comparative data in Fig. hat may explain why 5-HIAA is low-

TABLE III. PREVENTION OF THE LONG-TERM DEPLETING EFFECTS OF FENFLURAMINE AND NORFENFLURAMINE ON BRAIN SEROTONIN IN RATS BY FLUOXETINE, AN INHIBITOR OF UPTAKE INTO SEROTONIN NEURONS.[a]

Treatment group	Brain serotonin (μg/g)	
	Control	Fluoxetine-pretreated
Saline	0.61 ± 0.01	0.62 ± 0.01
Fenfluramine	0.44 ± 0.02*	0.61 ± 0.02
Norfenfluramine	0.44 ± 0.05*	0.64 ± 0.01

* Different from saline group, $P < 0.025$.
[a] Fenfluramine hydrochloride and norfenfluramine hydrochloride were injected at 15 mg/kg 1 hr after fluoxetine hydrochloride (10 mg/kg) and 1 week before groups of five rats were killed.

ered less rapidly than serotonin after fenfluramine (Table I). Though inhibition of tryptophan hydroxylation (Table II) (8) may contribute importantly to the *depletion* of serotonin stores by fenfluramine, the initial result of fenfluramine injection apparently is to *increase* the concentration of serotonin in the neural synapse by release and/or inhibition of reuptake (9, 10).

Our data showing decreased serotonin turnover 1 week after fenfluramine complement the observations of Clineschmidt *et al.* (11) showing decreased serotonin turnover, measured by the rate of 5-hydroxytryptophan accumulation following decarboxylase inhibition, 8 days after fenfluramine.

The actions of fenfluramine resemble closely those of *p*-chloroamphetamine on brain serotonin neurons in rats (see Table IV). Both drugs are actively transported into the serotonin neuron and then exert

multiple effects including release of serotonin, inhibition of serotonin synthesis, and depletion of serotonin content. The depletion of serotonin persists after single ip doses of both drugs for weeks or months. Duhault and Boulanger (27) recently reported that serotonin concentration in rat brain returned to normal within 48 hr after discontinuing long-term oral administration of fenfluramine. They interpreted their results as conflicting with those of Clineschmidt *et al.* (24) and of Harvey McMaster (25), who reported long-las depletion of serotonin, and considered differences in strains of rats might h been important. Strain differences seem likely to be involved, for long-lasting de tion of serotonin by fenfluramine has been observed in Wistar rats (this stu Sprague–Dawley rats from two diffe breeders (8, 26), Holtzman rats (25), (Carworth rats (24), and Charles River (11). A more likely basis of the differe in their results is the route of administra of the drug. We have found that daily administration of *p*-chloroamphetamin the diet of rats produces reversible de tion of serotonin (R. W. Fuller and oth unpublished data), whereas ip injec leads to irreversible effects (20).

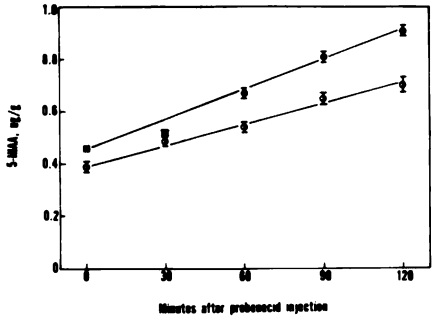


FIG. 2. Reduced turnover of brain serotonin measured by rate of 5-HIAA accumulation after probenecid in rats treated 1 week earlier with fenfluramine. Probenecid (200 mg/kg) was injected at zero time; fenfluramine hydrochloride (15 mg/kg) 1 week earlier. Solid dots indicate controls; half-filled circles indicate fenfluramine-pretreated rats. Mean values ± standard errors for five rats per group are shown.

Summary. Fenfluramine and its *N* methyl metabolite, norfenfluramine (3 fluoromethyl-amphetamine), lowered b serotonin, 5-hydroxyindoleacetic acid, tryptophan hydroxylase in rats acutely the effect persisted for more than 1 w after a single ip dose of the drugs. Serot depletion by fenfluramine and norfenfl mine was blocked by pretreatment fluoxetine, an inhibitor of uptake into s tonin neurons. Serotonin turnover, as m sured by the rate of 5-hydroxyindolea

TABLE IV. SIMILARITIES BETWEEN ip DOSES OF *p*-CHLOROAMPHETAMINE AND FENFLURAMINE IN TERMS THEIR EFFECTS ON BRAIN SEROTONIN NEURONS IN RATS.

Characteristic	*p*-Chloroamphetamine	Fenfluramine
Requires active uptake into the serotonin neuron, inasmuch as the depletion of serotonin is prevented by pretreatment with an uptake inhibitor	Meek *et al.* (12) Fuller *et al.* (13) Harvey *et al.* (14) Fuller and Molloy (7)	Ghezzi *et al.* (22) Garattini *et al.* (23) Clineschmidt *et al.* (11) This paper
Decreases not only serotonin but also other parameters associated with serotonin neuronal function – tryptophan hydroxylase activity, 5-HIAA concentration, and high-affinity uptake of serotonin	Sanders-Bush *et al.* (6) Sanders-Bush *et al.* (8) Pletscher *et al.* (15) Wong *et al.* (16) Miller *et al.* (17)	Garattini *et al.* (23) Clineschmidt *et al.* (11) Sanders-Bush *et al.* (8) This paper
Depletion of brain serotonin persists for weeks or months after a single drug dose	Sanders-Bush *et al.* (18) Sanders-Bush *et al.* (8) Fuller and Snoddy (19) Fuller *et al.* (20)	Clineschmidt *et al.* (24) Clineschmidt *et al.* (11) Sanders-Bush *et al.* (8) Harvey and McMaster (25) Sherman *et al.* (26) This paper
Causes histologic changes in the midbrain raphe region indicative on cell body degeneration	Harvey *et al.* (21) Harvey *et al.* (14)	Harvey and McMaster (25) Harvey *et al.* (14)

acid accumulation after probenecid administration, was decreased significantly still at 1 week after fenfluramine administration. The effects of fenfluramine and norfenfluramine on brain serotonin neurons in rats resemble those of *p*-chloroamphetamine and probably occur via similar mechanisms.

We are grateful to Dr. B. V. Clineschmidt for a prepublication copy of reference (11).

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Definition of the Cell Cycle Segment with Special Sensitivity to Vinblastine[1]

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Vinblastine sulfate, an alkaloid of the *Vinca rosea* group which is a potent inhibitor of cell division, has been used for many years as a cancer chemotherapeutic agent. It acts by inhibiting the assembly of microtubular proteins into spindle microtubules (1-3), producing a metaphase arrest similar to that caused by colchicine, that has been observed *in vitro* and *in vivo* (1, 2). In spite of the similarity of their effects on cells, the mechanism of action of vinblastine (Vb) and colchicine is probably different (3-5). Any drug or agent capable of prolonging the G2 phase or mitosis, permitting premature centriole replication and maturation of daughter centrioles, may induce multipolar spindles, leading frequently to cellular death, defective cytokinesis, and multinucleated tetraploid cells (6, 7). Irradiated cultured mouse cells (7) and Earle's L cells exposed to Vb (4) have been noted to have more than one pair of centrioles, and to undergo multipolar mitosis with abnormal chromosomal grouping (7) and multinucleation (4). We have found that exposure of HeLa cells to low concentrations of Vb produces similar effects, with high yields of multinucleated cells (8).

The purpose of this study was to define a discrete period in the cell cycle during which events take place that are especially sensitive to vinblastine, in the hopes of uncovering at least one cell cycle region which is critical for the formation of an intact mitotic spindle. This was approached by determining the period of the cell cycle during which Vb causes multinucleation.

Materials and methods. Cell culture. HeLa S 3 cells were grown in monolayers in 950-ml glass bottles in Eagle's minimal essential medium, supplemented with 10% activated fetal calf serum and 1% mine.

Cell volume and number were with an electronic particle counter Electronics Inc.) equipped with a nel particle size distribution a Model B.

Vb dose and time of exposure for multinucleation. Exponentially monolayers of HeLa cells were trea different concentrations of Vb (0 1.0 μg/ml of culture medium) for periods of time (30 min to 4 hr removal of Vb, the cell monolaye washed with warm phosphate-buff line and placed at 37° with fresh r At 2-hr intervals, for a period of mitotic cells were removed from flasks by gentle rocking of the medi the monolayers (9, 10) and seed sterile glass coverslips attached t rings, which were placed in petr (11). The concentration of cells coverslip ranged from 2 to 5 × 10^4/

The cells were allowed to attach grow on the glass for 20 to 24 h incubated at 37° in a humidified atm of 5% CO_2 in air. Then, the c were fixed in Carnoy fixative (3:1 acetic acid) and stained with 0.5% t blue (TB) for light microscopy obse The percentage of multinucleated c calculated by counting 600 to 120(different times and variable Vb co tions.

HeLa cell cycle analysis. The p the cell cycle (G1, S, G2) were t scoring the proportion of labele phases (12-14). The cells were g coverslips in petri dishes for 24 h undergoing a 30-min exposure to containing 0.5 μCi/ml (sp act, 7 Ci of tritiated thymidine. After expos radioactive medium was removed, cells were placed at 37° in fresh with an atmosphere of 5% CO_2 in

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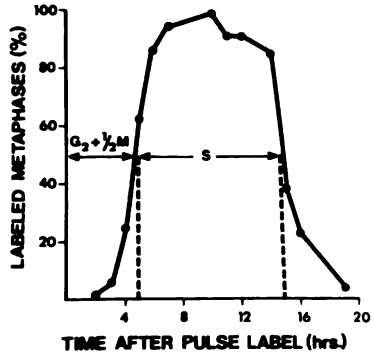
y intervals, for a period of 24 hr, slips with cell monolayers were re- d and prepared for autoradiography. uration of phases of the cell cycle was nined from the curve depicting the of appearance and disappearance of d mitoses (13) (Fig. 1). Mitotic figures phases) with four or more grains were lered labeled. Background grains were to one grain per cell cytoplasm. For coverslip, a minimum of 250 mitotic s was scored.

eled cells in metaphase first appeared en 2 and 3 hr (minimal G2 + 1/2 is) after exposure to tritiated thymi- One-half of the time for mitosis was l because mitoses were scored in me- se, which is about the midpoint of is. The frequency of labeled meta- cells rose to 98% at 10 hr after point maximal G2 + 1/2 mitosis). The aver- 2 + 1/2 mitosis was 4.5 hr as deter- l by the time between exposure and t which 50% of metaphase cells were d. At 19 to 20 hr the proportion of d mitoses declined 2.5%.

duration of S phase was obtained the difference between the two times ich 50% labeling occurred, and was to be 10 hr (Fig. 1).

eling of multinucleated cells. Expo- ally growing monolayers of HeLa cells treated simultaneously with 0.01 μg/ Vb and 0.5 μCi/ml of tritiated thymi- for a period of 30 min. After removal ese compounds by washing several



1. HeLa *S* phase of the cell cycle determined ing at hourly intervals the proportion of labeled cells (metaphases) after 30-min exposure to i/ml of [^{3}H]thymidine.

times with warm phosphate-buffered saline, the cultures were shaken at 2-hr intervals, for a period of 24 hr, to obtain mitotic cells, which were plated out on coverslips by the method described above for optimal multinucleation. One-half of the coverslips were processed for light microscopy and the other half for autoradiography.

Autoradiography. Coverslips were fixed in 3:1 ethanol–acetic acid at 0°, rinsed in ethanol, mounted cell-side-up on a microscopic slide and dipped in Kodak NTB 3 liquid emulsion (diluted 1:1 with distilled water at 43°). The slides were then exposed in a dry, dark atmosphere (sealed box containing desiccant) at 4° for 2 days. After exposure, the coverslips were developed in Kodak D-19 developer for 2 min at 20°, fixed in Kodak acid fixer, washed for 5 min in running water, dried, and stained through the emulsion with 0.5% TB.

Effect of inhibition of DNA synthesis on Vb-produced multinucleation. Randomly growing monolayers of HeLa cells were exposed to inhibitors of DNA synthesis (17, 18) [hydroxyurea (HU), 2 m*M*) and cytosine arabinoside (Ara C, 10 μg/ml)] for a period of 1 hr. During the second half-hour of exposure, 0.01 μg/ml of Vb was added. Simultaneous controls were exposed only to Vb in all experiments. Afterward, the cells were washed and incubated with fresh medium. At 2-hr intervals, until 6 hr had elapsed from removal of the inhibitors, mitotic cells were collected by shaking the cultures, seeded, incubated for 20–24 more hr, and then fixed.

Results. The ability of Vb to produce multinucleation in HeLa cells was used to define a period of the cell cycle which determines the successful outcome of subsequent mitosis. This was accomplished by determining the period of the cell cycle of maximal sensitivity to Vb.

Maximal multinucleation [16 ± 8%(SD)] was found when cells were exposed to 0.01 μg/ml of Vb for 30 min, starting at 5 hr 26 min before mitosis (Table I), a period which is in late S, but close to the S-G2 boundary of most cells in these cultures. The majority of the multinucleated cells had 2 to 6 nuclei (Fig. 2), but cells with 10 to 18 were not infrequent. Simultaneous exposure to Vb and tritiated thymidine showed that DNA

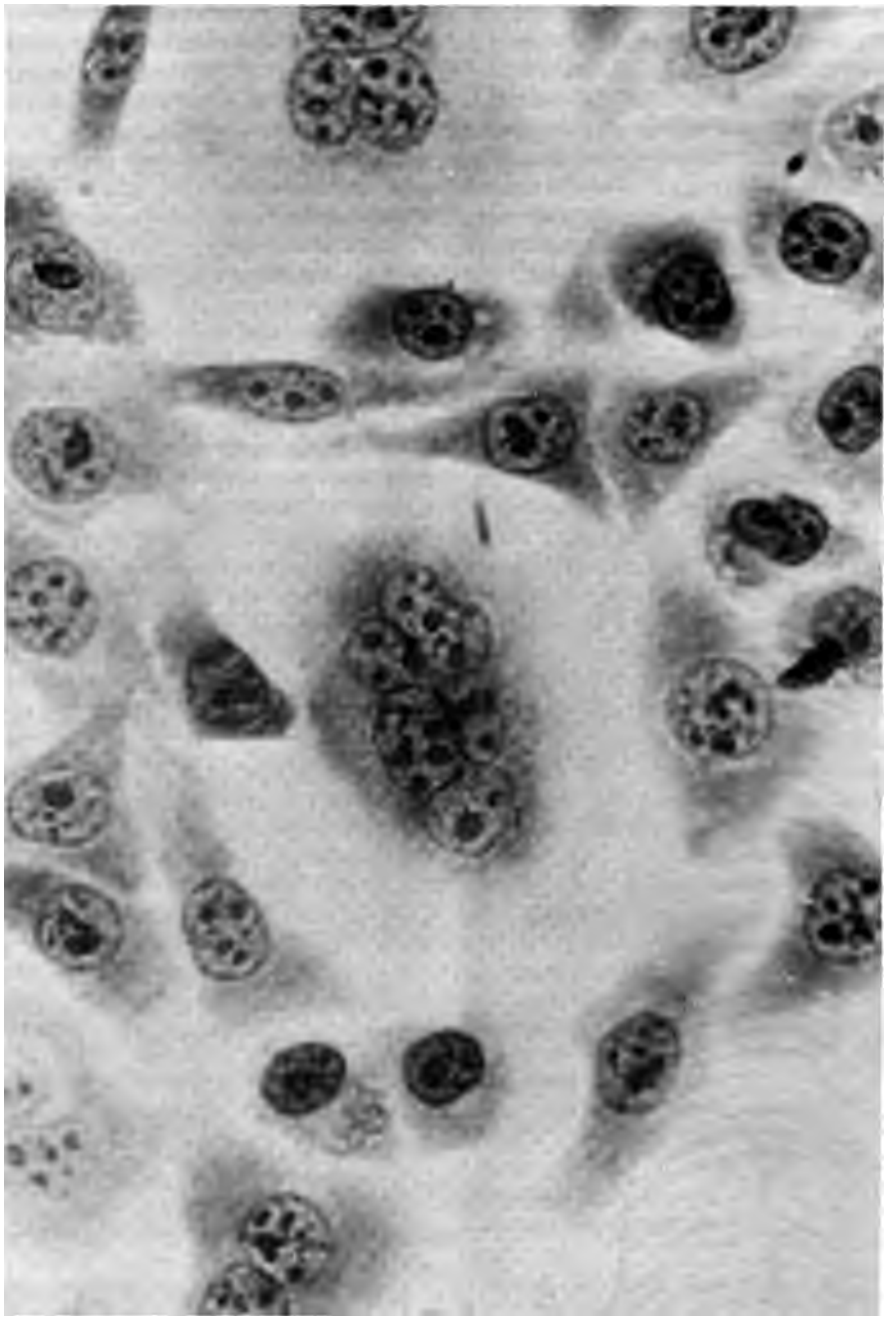


FIG. 2. Multinucleated cell obtained after 30-min exposure to 0.01 μg/ml of vinblastine, 6 prior to mitosis, and fixed 20 hr later. Toluidine blue, × 400.

synthesis was taking place during the period in which Vb produced multinucleation. It was observed that 100% of the multinucleated cells incorporated the radioisotope, but the intensity of autoradiographic labeling of multinucleated cells was less than that mononuclear cells. This might have curred because Vb-affected cells were th members of the culture which were trav ing the end of the DNA synthetic phas

I. DOSE-RELATED VINBLASTINE-INDUCED LTINUCLEATION (0.5-hr EXPOSURE).

l)	Peak time[a]	Maximal multinucleation (%)
	2 hr	8
	6 hr	10
	5 hr, 26 min	16
	6 hr	13.0
	4 hr, 30 min	14
	4 hr	12
	6 hr	11
	6 hr	7

l mean, 5 hr, 6 min ± 1 hr, 34 min (SD).
ınd maximal multinucleation represent av-
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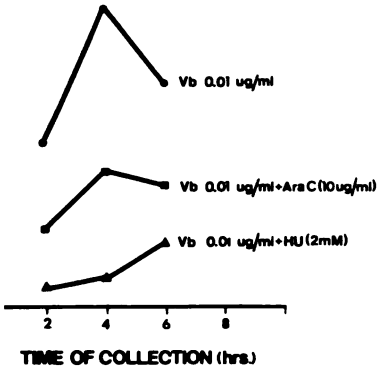

Exponentially growing monolayers of were exposed to vinblastine (Vb), Vb + abinoside (Ara C), and Vb + hydroxyurea l hr. After removal of the drugs, mitotic collected, seeded on coverslips, and incu-0–24 hr. After this period, coverslips were xamined microscopically for determination ge of multinucleated cells.

ycle, and the period of exposure to isotope included only a few min-he S phase, or because Vb inter-h the uptake of the isotope.

ient with inhibitors of DNA syn-rkedly reduced the multinucleation by Vb (Fig. 3).

sion. The sequential events which cell reproduction are poorly under-ie main landmarks are DNA syn-d mitosis, but it is obvious that a nber of processes must occur in rder to ensure cell division. It is ell established that histone synthe-sis is coupled to DNA synthesis (15, 16, 19), and it would also appear that mitotic spindle formation takes place during early stages of mitosis (20, 21). It seems reasonable to assume that events preparatory for the spindle must precede formation of the visible spindle. Work reported here suggests that events crucial for orderly assembly of the spindle microtubules take place shortly before replication of the chromosomes is completed. This would explain the observed maximal disruptive effect near the S-G2 boundary. This conclusion is supported by the inhibitory effect of hydroxyurea and cytosine arabinoside, since some DNA synthesis appears to be necessary for the Vb-sensitive microtubule formative events to take place. It would appear, therefore, that the last period of chromosome replication is accompanied by events which are preparatory for microtubule assembly and are exquisitely sensitive to Vb.

Summary. The ability of Vb to produce multinucleation in HeLa cells was used to define a period of the cell cycle with special sensitivity to vinblastine. Exponentially growing monolayers of HeLa cells were treated with Vb for 30 min. After removal of Vb, mitotic cells were collected, seeded on coverslips, and examined microscopically. Maximal multinucleation was found when the cells were treated with Vb about 5 hr before mitosis, the period of time which corresponds to the late S, close to the S-G2 boundary of these cells. Simultaneous exposure to Vb and [^{3}H]thymidine showed that DNA synthesis was taking place during the period of maximal sensitivity to Vb, and treatment and inhibitors of DNA synthesis abolished this Vb effect. These findings indicate that events take place near the end of the S phase which affect the integrity of the spindle at the subsequent mitosis.

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Bone Mineralization during a Developing Zinc Deficiency[1,2] (40023)

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ey and Swenerton (1) reported that nt adult rats were unable to release nt quantities of bone zinc to meet nands of the developing fetus during ay period of dietary zinc deficiency ncluded that in the mature skeleton n bone is not available to the rat i the face of demonstrated need." er, more recently Harland *et al.* (2) ed that bone may store zinc con- in excess of the requirement and s zinc may be available for utilization a subsequent period of zinc deprivа- he studies used young, immature se quail. The purpose of the reported nent was to monitor bone and zinc and to determine if the net zinc of bone was altered during the pment of a zinc deficiency in the growing rat.

rials and methods. One hundred weanling (45-50 g) Sprague-Daw- were housed in individual stainless- ges free of solder. Deionized water 5 ppm zinc) was provided *ad libitum.* imals were divided into three experi- groups of comparable body weight l the following diets: zinc sufficient, *um;* zinc deficient, *ad libitum;* and fficient, pair-fed by restricting the daily intake to that of the zinc- nt group.

The composition of the basal diet has been described elsewhere (3). The basal diet (1.5 μg of Zn/g of diet) was supplemented with $ZnCO_3$ to give a total of 93 mg of Zn/kg for the zinc-sufficient diet.

Upon arrival, six rats were sacrificed to provide baseline values. Four rats in the zinc-sufficient, *ad libitum* group were sacrificed on Days 9 and 13, while three were sacrificed on Day 23. Four rats in the pair-fed zinc-sufficient group were sacrificed twice weekly. Six rats in the zinc-deficient, *ad libitum* group were sacrificed on Days, 1, 2, and 3 of the experiment and, thereafter, twice weekly.

Animals were sacrificed under light ether anesthesia. Blood was collected in polyethylene syringes with stainless-steel needles and polypropylene hubs. Two drops of a 30% sodium citrate solution were added to each sample as an anticoagulant. Any hemolyzed samples were discarded. The right femur was removed, stripped of tissue, and dried to a constant dry weight in a vacuum oven at 70°.

Plasma and bone for all animals were analyzed for zinc using atomic absorption spectrophotometry after acid digestion using a mixture of $HClO_4$ and H_2O_2 (3). Bone from animals sacrificed at 0, 9, 16, and 23 days of the experiment was analyzed for calcium by atomic absorption spectrophotometry[6] and phosphorus by the colorimetric method of Fiske and Subbarow (4).

Results. Figure 1 shows the weight gain of all three groups of rats. After the second day of treatment the zinc-deficient group displayed a retarded growth rate, and after 3 days there was little increase in weight for the remainder of the experiment. The zinc-sufficient pair-fed group grew at a rate intermediate to the other groups.

e of these data were presented at the Fifty- nual Meeting of the Federation of American for Experimental Biology, Atlantic City, New April 13-18, 1975.

animals were maintained in animal care facil- edited by the American Association for Ac- n of Laboratory Animal Care.

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es River Breeding Laboratories, Wilming- achusetts 01887.

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Plasma zinc (Fig. 2) for the zinc-deficient group dropped 55% (107 to 48 μg/ml, $P < 0.001$) after 1 day of dietary deficiency. The plasma zinc decreased an additional 16% (to 31 μg/ml, $P < 0.001$) by Day 3, for a total of 71% (107 to 31 μg/ml, $P < 0.001$), and then remained relatively constant for the rest of the experiment. The plasma zinc of the zinc-sufficient and pair-fed controls remained in the normal range throughout the experimental period.

Femur concentration of zinc in the zinc-deficient group decreased steadily until Day 13 (Fig. 3), when the mean level was 61% (150 to 58 μg/g dry weight, $P < 0.001$) less than baseline. In contrast, the sufficient and pair-fed groups increased their femur zinc concentrations from the baseline. The mean total zinc content of the femurs from the zinc-deficient rats decreased 21% (11.7 to 9.2 μg/femur, $P < 0.05$) by Day 3 and then decreased more gradually to an overall decrease of 37% (11.7 to 7.4 μg/femur, $P < 0.01$) on Day 23. The zinc-sufficient rats showed a steady increase in the total zinc content of their femurs. The pair-fed group also increased its total femur zinc, though at a lesser rate. As the experiment progressed, the femurs increased in weight for all groups, including the zinc-deficient rats (Fig. 4). However the increase was least for the zinc-deficient group, while the pair-fed group had an intermediate gain in femur weight.

Table I shows that although the total and concentration of calcium and phosphorus increased in the femurs, regardless of treatment, the calcium to phosphorus ratio remained relatively constant over the dietary period. All three groups had radios ranging between 1.80 and 2.03. However, the calcium and phosphorus accumulation in fe-

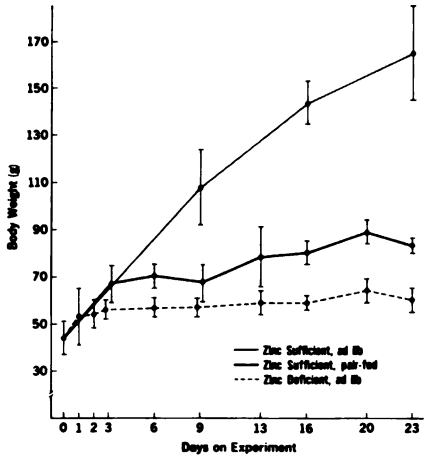


FIG. 1. Body weight during the development of a zinc deficiency.

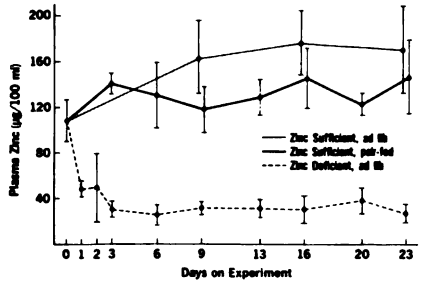


FIG. 2. Plasma zinc during the development of a zinc deficiency. Hemolyzed samples were discarded. Each point represents four to six samples, except for three samples on Day 23 of zinc-sufficient, *ad libitum* diet.

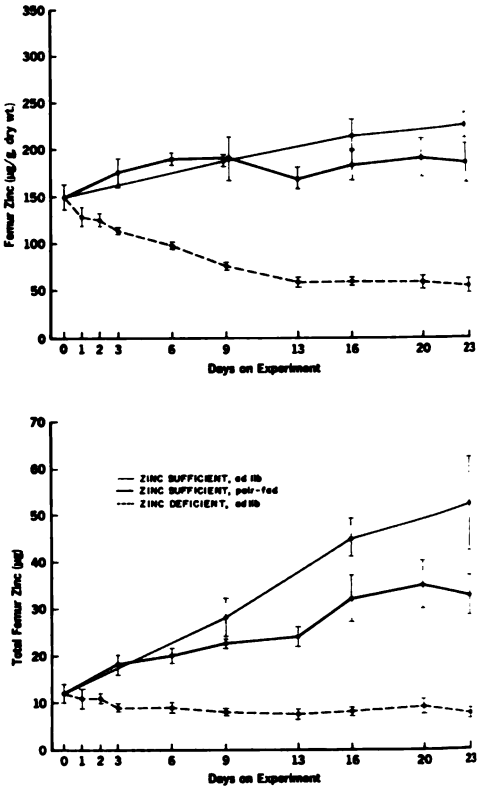
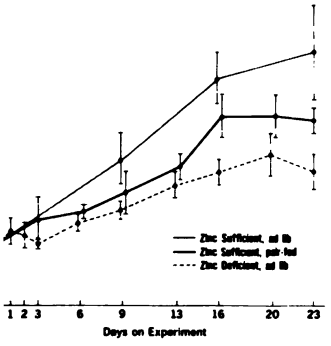


FIG. 3. Femur zinc concentration and total femur zinc during the development of a zinc deficiency.

: zinc-deficient group was signifi- ($P < 0.05$) than that in the other the end of the experimental pe-

on. The present data are in agree- previous reports that during the an acute zinc deficiency, plasma ses drastically over the first 24 hr ues to decline for the next 2 days rthermore, in this experiment ttle body weight gain after 3 days. : concentration declined steadily 3th day of the deficiency. These neters, body weight, plasma, and entration, are commonly used to status and clearly indicate a zinc-



:mur weight during the development of a y.

deficiency state by the end of the experimental period. However, the time course of the decline was different for each parameter. Plasma zinc responded quickly to the dietary zinc inadequacy, whereas femur zinc concentration decreased slowly. This may indicate most accurately the gradual deterioration of the overall zinc status of the animal.

The total femur zinc of the zinc-deficient group decreased 37% over the experimental period. Extrapolation of the femur data to the whole skeleton suggests that an appreciable net amount of zinc was lost or released during the developing zinc deficiency in these young growing rats. However, the released zinc was not available and/or insufficient to prevent the eventual zinc deficiency. The total net quantity of skeletal zinc released can not be estimated from our data since the different types of skeletal bone may have zinc concentrations differing from that of the femur. Also, zinc may be released at different rates for different bones.

Our data indicate that some zinc in bone is released during a zinc deficiency. This supports the results of Harland *et al.* (2). Both investigations used immature animals, whereas Hurley *et al.* (1) concluded that zinc was not released from the skeleton of

LE I. FEMUR CALCIUM AND PHOSPHORUS DURING THE DEVELOPMENT OF ZINC DEFICIENCY.

	Number of samples	Calcium		Phosphorus		
		(mg/femur)	(mg/g dry wt)	(mg/femur)	(mg/g dry wt)	Ca:P
0	6	12.4 ± 2.7*	159 ± 20	6.5 ± 1.6	83 ± 13	1.92 ± 0.11
itum	4	29.1 ± 3.7[a]	197 ± 10[a]	15.6 ± 2.6[a]	105 ± 5[a]	1.88 ± 0.18[a]
d	4	23.3 ± 2.8[b]	193 ± 12[a]	12.1 ± 1.5[b]	101 ± 9[a]	1.92 ± 0.07[a]
itum	6	20.1 ± 1.1[b]	188 ± 14[a]	10.3 ± 0.7[b]	97 ± 8[a]	1.95 ± 0.15[a]
tum	4	43.4 ± 4.5[a]	207 ± 7[a]	21.7 ± 1.8[a]	104 ± 1[a]	2.00 ± 0.06[a]
d	4	34.4 ± 3.6[b]	195 ± 3[b]	17.5 ± 2.1[b]	99 ± 4[a]	1.97 ± 0.08[a]
itum	6	25.5 ± 1.6[c]	188 ± 9[b]	12.6 ± 1.1[c]	93 ± 4[b]	2.03 ± 0.09[a]
tum	3	52.8 ± 8.3[a]	228 ± 9[a]	28.1 ± 3.9[a]	121 ± 4[a]	1.88 ± 0.04[a]
d	4	38.8 ± 3.8[b]	221 ± 11[a]	21.5 ± 1.9[b]	123 ± 5[a]	1.80 ± 0.03[a]
itum	6	27.6 ± 2.8[c]	199 ± 9[b]	14.9 ± 1.4[c]	107 ± 5[b]	1.86 ± 0.05[a]

nc sufficient; ZnD = zinc deficient.

ndard deviation; means within the same treatment day with different superscripts are significantly : 0.05. The data were subjected to one-way analysis of variance and the means compared using tiple range test (5).

/ 23 all calcium and phosphorus parameters were significantly different from their respective s ($P < 0.05$). The Ca:P ratios were not significantly different from the baseline.

older pregnant rats. Thus, a major factor in the release of bone zinc during periods of deprivation appears to be the stage of skeletal maturity. The kinetics and mechanisms of the zinc release still need to be defined.

Although the total femur zinc decreased 37%, the femur zinc *concentration* decreased by 61%. This discrepancy can be partially explained by changes in the femur weight. The femur continued to increase in weight during zinc deficiency, although at a decreased rate compared to the control groups. Thus, the large decrease in bone zinc concentration could be, in part, due to a "dilution" resulting from the increased femur weight. In addition, the decrease in total zinc contributed to the decreased concentration. The femurs increased in weight during a period of a decrease in body weight as the deficiency developed. We did not measure femur length and thus were unable to determine if linear growth contributed to this weight gain of the femurs.

The femur weight gain for the zinc-deficient group appears to be due to an increase in bone mineralization. Both the total and concentration of calcium and phosphorus increased as the deficiency developed while, concomitantly, zinc (total and concentration) was decreased. Furthermore, the Ca:P ratio remained relatively constant. Therefore, the decrease in zinc appears to be independent from calcium and phosphorus in the bone. Thus, zinc may not be necessary in a precise ratio to calcium or phosphorus, but rather as a cofactor or catalyst in the mineralization process.

Summary. Alterations in bone mineralization were examined during the progression of a zinc deficiency in rats. One hundred rats were divided into three groups and fed the following diets: zinc sufficient, *ad libitum;* zinc deficient, *ad libitum;* and zinc sufficient, pair-fed the daily intake of the deficient group. Plasma zinc concentration of the zinc-deficient group decreased dramatically during the first 24 hr of deficient diet and continued to decline over the next 2 days. Body weight gain essentially stopped after 1 week. During the experimental period of 23 days, femur zinc concentration decreased 61%, while total femur zinc decreased 37%. Yet, both the total and concentration of calcium and phosphorus increased and the Ca:P ratio remained relatively constant. The present data indicate that zinc can be released from bone during periods of dietary zinc deficiency. Furthermore, the decrease in zinc appears to be independent from calcium and phosphorus in the bone.

The authors thank M. P. Howard for her excellent technical assistance and Dr. N. R. Calhoun for helpful discussions.

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ıce of Malnutrition on the Concentration of IgA, Lysozyme, Amylase, and Aminopeptidase in Children's Tears[1] (40024)

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ı–calorie malnutrition (PCM) sig- increases susceptibility to infec- ıildren (1). This may be caused by ired cellular immunity often ob- PCM children (2) or by suppres- ımoral immunity and its associated phagocytosis and complement (3). , malnourished children often have r increased levels of serum immu- ns (4, 5). Most initial infections or near mucosal surfaces which cted from pathogens by secretory to a lesser extent IgM, IgG, and (6). A reduction in the relative ıtion of IgA in the total protein rom the nasopharyngeal surfaces reported in severely malnourished (4). In this paper we report the moderate as well as severe PCM dified tears (lacrimal secretions). ations of locally produced IgA, , and amylase are compared to rived components in tears.

ıls and methods. Study population. : children of both sexes were re- ır the study in two health centers Colombia. The children ranged in 2 to 5 years, with a mean of 3.75 l came from the same uniformly ıeconomic background. Informed vas obtained from the mother of d prior to recruitment into the ı the days that the samples were ch child was examined thoroughly ıtrician (MAR) for signs of disease liarrhea, cough, skin eruptions, etc.). Children with overt clinical signs were not sampled. The nutritional status of each child was determined by physical examination and weight-for-height using standard curves for Colombian children developed by the Instituto Colombiano de Bienestar Familiar (Colombian Institute of Family Welfare). Since such athropometric indexes are influenced by genetic differences between populations, it is important to use a standard curve which has been developed in the population from which the study sample is drawn. The ICBF weight for height scale has been found to assign a nutritional status comparable to that obtained using the widely accepted Gomez classification (7). Using this scale, Grade I children fall into 80–90%, Grade II into 70–80%, and Grade III less than 70% of the normal standard. By these criteria, 27 of the children were normal, 11 were Grade I, 9 were Grade II, and 17 were grade III malnourished.

Immunoglobulin and enzyme assays. Serum was stored at −20°. Tears were collected in 2 × 5 × 2-mm sterile sponges (Weck cel, E. Weck and Co., N.Y.) that were placed between the eyeball and the lower conjunctiva for 30–60 sec. The fluid was extracted from the wet sponge by compression into the bottom of a small polyethylene tube with a capillary pipet. A sample was taken from each eye on two different occasions 3–5 days apart. The tears were pooled and stored at 4°.

Serum IgG, IgA, IgM, and IgD were measured by radial immunodiffusion (8) using monospecific antisera (Meloy Laboratories, Springfield, Va.). IgA and IgG were quantitated in tears using commercial immunodiffusion plates for low concentrations (Hyland, Costa Mesa, Calif.). Human secretory IgA isolated from saliva (gift of Hyland Company) has a 45% lower measure level than its known concentration. Therefore the concentration of IgA obtained with the serum IgA standards was

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multiplied by 2.1 to give the concentration of dimeric secretory IgA (S-IgA). Total protein in serum and secretions was measured by the technique of Lowry *et al.* (9), modified to require only 1 μl of sample. Albumin in tears was quantitated using low-level immunodiffusion plates (Hyland). Serum albumin was measured by the method of Doumas *et al.* (10). Lysozyme (muramidase) activity in tears was measured based on hydrolysis of *Micrococcus lysodeikticus* in agar plates (11). Purified hen's egg lysozyme was used as a standard. Lysozyme values were multiplied by a factor of 2, as Bonavida *et al.* (12) have shown that the specific activity of human lysozyme is twice that of hen's egg lysozyme used as the standard. Methionine aminopeptidase activity was measured in 1 μl of tears using a modified assay for aminopeptidase in bacteria (13) by hydrolysis of 0.4 ml of 10^{-4} *M* L-methionyl β-naphthalamide in 0.1 *M* Tris-HCl buffer (pH 8.0) at 37° for 3 hr. The amount of fluorescence produced by hydrolysis of the substrate was determined with a Corning 7-60 excitation filter and Wratt 47B secondary filter. Fluorescence obtained was compared to a control solution of 10^{-4} *M* β-naphthalamide hydrolyzed/hr at 37°. Amylase activity in tears was measured by the assay of Somogyi (14). One Somogyi unit is defined as the amylase activity required for the formation of reducing power equivalent to 1 mg of glucose in 30 min at 40°.

Results. Age, sex ratio, and hemogram values. The mean ages, distribution of males and females, and hemogram results for the four groups of children are shown in Table I. The ages were similar in the four groups, although the Grade II malnourished children averaged 12% (5 months) younger than the normal children. There were nearly twice as many males as females in all groups. No evidence of anemia was observed even in the most severely malnourished children. Total and differential leukocyte counts were uniform, with somewhat elevated total counts in all malnourished children.

Immunoglobulins and enzymes in serum. Table II contains the values obtained for four immunoglobulins, total protein, albumin, and aminopeptidase in the sera of the four groups of children. Significantly elevated levels of IgA were observed in the most severely malnourished group, while concentrations of IgG, IgM, and IgD were not influenced by nutritional status. Serum albumin was significantly reduced ($P < 0.05$) in both Grade II and Grade III children, confirming the diagnosis of severe malnutrition of the Marasmus-Kwashiorkor type in these groups. Aminopeptidase was

TABLE I. AGE, SEX DISTRIBUTION, AND HEMOGRAM VALUES IN NORMAL AND MALNOURISHED CHILDREN.

	Normal	Grade I	Grade II	Grade III
Number of children	27	11	9	17
Age (months)	43 (30-57)[a]	44(27-60)	38(27-48)	45(25-60)
Sex ratio (F/M)	10/17	4/7	3/6	6/11
Hemoglobin (g%)	12.7 ± 0.8[b]	12.3 ± 0.5	12.2 ± 0.3	12.2 ± 2.8
Hematocrit (%)	39 ± 1	37 ± 1	38 ± 1	38 ± 1.9
Leukocytes (per mm^3)	10,047 ± 2123	12,190 ± 1950	11,844 ± 1333	11,580 ± 1925

[a] Mean (range).
[b] Mean ± standard error of the mean (SEM).

TABLE II. IMMUNOGLOBULINS AND OTHER SERUM PROTEINS IN NORMAL AND MALNOURISHED CHILDREN.

	Serum immunoglobulins (mg%)						
Nutritional status	IgA	IgG	IgM	IgD	Aminopeptidase[a]	Albumin (g%)	Total protein (g%)
Normal (27)	91 ± 8[b]	1089 ± 66	131 ± 11	7.5 ± 1.3	0.93 ± 0.07	4.61 ± 0.13	5.52 ± 0.25
Grade I (11)	82 ± 10	871 ± 72	97 ± 8	5.4 ± 1.6	0.98 ± 0.09	4.34 ± 0.20	5.42 ± 0.23
Grade II (9)	107 ± 18	1022 ± 107	131 ± 26	7.6 ± 2.5	0.98 ± 0.09	2.89 ± 0.21[c]	4.65 ± 0.36
Grade III (17)	122 ± 15[c]	855 ± 69	123 ± 9	7.7 ± 2.1	0.89 ± 0.10	2.70 ± 0.08[c]	3.85 ± 0.17[c]

[a] Enzyme activity expressed as units (micromoles of substrate hydrolyzed per hour at 37°).
[b] Mean ± SEM.
[c] Significantly different from normal children at the 5% confidence level.

found in similar concentrations in all children. The values of all serum proteins for the normal and the marginally malnourished children, Grade I, were similar.

Immunoglobulins and enzymes in tears. Table III contains the results obtained in the tears of the study children. In contrast to its elevated concentration in the serum, S-IgA was found at significantly reduced levels in the tears of Grade III children. Moderately malnourished children (Grades I and II) also tended to have lower concentrations of S-IgA and amylase than control children, although the differences were not statistically significant. Lysozyme levels and amylase activity were also significantly depressed ($P < 0.05$) in Grade III children. The values observed for total protein, albumin, and aminopeptidase in tears were unaffected by the child's nutritional status.

Linear regression analyses were performed on selected pairs of variables in tears and serum. No correlation was found between the concentration of IgA in serum and tears ($r = 0.07$, $P < 0.10$) or between levels of S-IgA and albumin in tears ($r = 0.38$, $P < 0.10$). The correlation between IgG in serum and tears was also not significant ($r = 0.16$, $P < 0.10$), while the correlation between lysozyme and S-IgA was significant ($r = 0.824$, $P < 0.05$).

Discussion. We have demonstrated reduced concentrations of S-IgA, amylase, and lysozyme in the tears of severely malnourished children. Our results in Colombia confirm the previous finding that S-IgA is decreased in the nasopharyngeal secretions of Thai and Indian children with PCM (4, 15). In addition, the synthesis or secretion of other locally produced proteins, lysozyme, and amylase into tears was impaired. Decreased activity of lysozyme has been observed in leukocytes of children with PCM (16). Reduced secretory concentrations of S-IgA and to a lesser extent, lysozyme, are potentially important factors in the defense of mucosal surfaces. The fact that total protein, IgG, aminopeptidase, and albumin in tears were not influenced by nutritional status indicates that the reduced levels of S-IgA, amylase, and lysozyme were not due to reduced volume of secretion in children with PCM, as has been suggested by experimental studies with saliva in malnourished rats (17). It appears that the mechanism involves an impairment in the local synthesis and/or secretion of these proteins.

Chandra has observed that concentrations of both S-IgA and specific antibodies to polio and measles vaccination are reduced in malnourished Indian children (15). We have confirmed these findings with respect to S-IgA in an unmodified secretion (tears) from Latin American children. Thus, it appears that malnutrition may significantly reduce both unstimulated levels of S-IgA and antibody produced to a specific antigenic challenge. These two events could have a profound influence on local immunity to an infectious agent: the former by allowing an organism to establish itself more easily on the mucosal surface and penetrate the epithelial barrier, resulting in increased infection rates; the latter by reducing the effectiveness of the immune response in dislodging an established infection, resulting in increased morbidity and prolonged recovery periods. The reduction of S-IgA, amylase, and lysozyme, which are specifically not transudated from the serum, may reflect a change in the secretory capability of the

TABLE III. AMYLASE ACTIVITY AND IMMUNOGLOBULIN LEVELS IN TEARS OF NORMAL AND MALNOURISHED CHILDREN.

Nutritional status	Amylase activity[a]	S-IgA (mg/dl/g of protein)	Lysozyme (mg/dl/g of protein)	Aminopeptidase activity[b]	Protein (g/dl)	Albumin (mg/dl)
Normal (27)	376 ± 82	49 ± 8	103 ± 18	0.58 ± 0.18	1.39 ± 0.18	34 ± 8
Grade I (11)	322 ± 52	43 ± 6	119 ± 16	0.76 ± 0.22	1.18 ± 0.16	53 ± 9
Grade II (9)	252 ± 43	31 ± 7	53 ± 9*	0.57 ± 0.16	1.63 ± 0.08	46 ± 11
Grade III (17)	199 ± 45*	27 ± 6*	62 ± 14*	0.59 ± 0.13	1.34 ± 0.17	32 ± 6

[a] Somogyi units per gram of protein ± SEM.
[b] Units (micromoles hydrolized per hour at 37°) per gram of protein.
* Significantly lower than normal children at the 5% confidence level.

mucosal surface and/or local protein synthesis. In addition, a combined reduction in immunity to some mucosal organisms such as *Neisseria gonorrhoeae* may occur since both S-IgA and lysozyme are affected. IgA antibody responses to an antigen lack the elevated secondary response memory seen in the IgG antibody response (18). Therefore, the depressed levels of S-IgA we observed in these children's secretions may represent significantly depressed secretory immunity to pathogens with which they have previously been infected as well as new pathogens infecting the mucosal surfaces. It has been shown that the S-IgA response following a single gonococcal infection which is eliminated by treatment rapidly decreases to almost undetectable levels at 4–5 months (18). Levels of S-IgA and albumin were not correlated in tears, a finding similar to that already reported in nasopharyngeal washings (4, 15). IgA synthesis in general did not seem to be impaired, since IgA was actually elevated in the serum of these same children in whose tears its concentration was suppressed. The marked contrast between elevated serum and depressed secretory IgA which we observed has been reported recently by others (4, 5). The lack of significant influence of nutritional status of IgG, IgM, and IgD concentrations in serum confirms the findings of others (4, 5). The lower levels of amylase, lysozyme, and S-IgA in tears of moderately malnourished children, although not statistically significant as in severely malnourished children, suggest that their mucosal immunity is also reduced even though they are not as nutritionally deprived.

The mucosal surfaces of the eyes and respiratory and gastrointestinal tracts serve as the portals of entry for nearly all infectious agents. Since malnourished children have increased infections of mucosal surfaces, such as gastrointestinal and ocular infections, increased antigenic stimulation of mucosal surfaces could be expected, with higher levels of S-IgA antibodies and total S-IgA rather than the lower levels we observed. The influence of renutrition on suppressed levels of S-IgA needs to be further defined as well as a clearer understanding of the mechanism of this suppression and its effects on the mucosal immune response to new antigens.

The mechanism(s) by which PCM influences S-IgA levels may be related to deficiency of dietary protein, vitamins, or trace minerals resulting in abnormal morphology or function of epithelial cells. Genetic deficiency of secretory component has been found to be associated with normal serum IgA levels with no IgA in the secretions (19). Similarly, patients with common variable hypogammaglobulinemia or selective IgA deficiency appear to have a defect in the binding of secretory component to IgA (20). We observed decreased secretory IgA concentrations in the presence of increased serum IgA levels in malnourished children, which could have resulted from impaired production and/or binding of secretory component without a reduction in the synthesis rate of IgA. Thus, increased antigenic stimulation of the mucosa due to recurrent respiratory and gastrointestinal infections in malnourished children could result in increased production of IgA which is incapable of getting through the epithelial barrier or remaining in the secretion intact.

Our observations are at variance with the elevated levels of S-IgA reported in intestinal washes from malnourished Indonesian children (21). This discrepancy is difficult to explain based upon the data available. It may be related to differences in the type of secretion samples or, more likely, to the nature of the nutritional deficiencies which accompany reduced protein and calorie intake in the two populations. It is quite possible that the presence or absence of concomitant zinc deficiency, for example, could result in the differences observed in the two studies (22). Since data on trace mineral or vitamin deficiencies are not available from either study, their relative importance remains to be evaluated.

Our findings indicate that the increased susceptibility of malnourished children to infections of mucosal surfaces may be explained, in part, by a generalized decrease in the concentrations of protective substances locally produced to protect those surfaces. Given the delicate balance which often characterizes host–parasite interac-

tions, it is not unreasonable to suggest that the decreases of 40–50% which we observed in the levels of IgA and lysozyme in the tears of malnourished subjects may be sufficient to upset the balance in favor of the pathogen.

Summary. Sixty-four pre-school-age children from a poor urban population in Colombia were studied to determine the influence of moderate to severe malnutrition on several proteins in the serum and tears. Secretory IgA, lysozyme, and amylase were found in significantly reduced concentrations in the tears of malnourished children. No influence of nutritional status on total proteins, albumin, and aminopeptidase was observed in this secretion. In the serum, IgA concentrations were significantly higher in the most malnourished group, while total proteins and albumin were reduced. These data suggest that increased susceptibility to infections of mucosal surfaces in malnourished children may be due to reduced concentrations of protective substances such as IgA and lysozyme in secretions.

The fine technical assistance of Marta McMurray, Ana Carlina de Aly, Cora Foulks, and Joan Roberts is greatly appreciated. We are indebted to Gail Wilkerson for assistance with data analysis.

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Blastokinin Secretion, Ovarian Activity, and Embryo Survival after Melengestrol Acetate in Rabbits[1] (40025)

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Blastokinin (uteroglobin) comprises approximately 40-50% of the total protein in rabbit uterine fluid during the days around the time of implantation (1-3). In ovariectomized rabbits, the concentration of blastokinin in uterine flushings increases dramatically after progesterone treatment (4, 5), but only slightly if estrogen is given (6). Metabolites of progesterone also stimulate blastokinin secretion (7), as do some oral contraceptive steroids (8).

However, the physiological significance of this protein has yet to be determined. Administration of melengestrol acetate (MGA), a synthetic progestogen, lowered fertility in rabbits, allegedly through altering the time course of uterine protein secretions (9). In an attempt to elucidate further the possible functions of blastokinin in embryo survival in rabbits, we studied the effects of melengestrol acetate (MGA) administration on blastokinin secretion and ovarian activity. The ability of supplemental blastokinin to increase implantation rate of transferred rabbit embryos was examined under conditions known to reduce fertility.

Materials and methods. General procedures. A series of four experiments was conducted. In the first experiment, progesterone and estradiol were measured in blood samples collected prior to and during MGA or corn oil treatment and during a subsequent period of pseudopregnancy induced by treatment with human chorionic gonadotropin (HCG). In the second and third experiments, blastokinin was assayed in uterine flushings collected from rabbits sacrificed during MGA or corn oil treatment or during the period of induced pseudopregnancy which followed. In the final experiment, embryos collected from superovulated donors were transferred in medium containing blastokinin or rabbit blood serum albumin to pseudopregnant recipients which had been treated with MGA or corn oil. Significance of differences between treatments was analyzed by paired *t* test.

Treatment procedures. In all four experiments, treatments consisted of daily injections (10 days) of either 10 μg MGA/kg body weight dissolved in corn oil or corn oil alone. Pseudopregnancy was induced by intravenous administration of 100 IU of human chorionic gonadotropin[3] on the third day following MGA or corn oil treatment (9).

Serum and uterine fluid samples. In the first experiment, blood samples were collected on alternate days from a marginal ear vein of six corn oil- and six MGA-injected mature New Zealand white female rabbits. Samples were collected from 12 days pretreatment until Day 7 of pseudopregnancy. For three rabbits in each treatment group, sampling began on the first day and continued on odd-numbered days, whereas the remaining rabbits were sampled on even-numbered days. Serum was obtained by centrifugation of whole blood after it had been allowed to clot at room temperature. Samples were stored frozen until assayed for progesterone and estradiol.

In the second experiment, uterine flushings were obtained from mature does on Days 1 to 9 of pseudopregnancy (three does/treatment/day) following 10 days of MGA or corn oil injections. In the third experiment, uterine flushings were recovered (three does/treatment/day) from

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ı MGA or corn oil and sacrificed
), 6, and 9 of treatment or on the
ay following the last injection.
ɛ sacrificed by cervical dislocation,
ri were excised immediately,
of fat, rinsed with tap water, and
remove any traces of blood. Each
ornus was flushed with 3 ml of
sodium chloride. Flushings were
ɛd at 700*g* to remove cellular de-
hose obviously contaminated with
re discarded. Samples were stored
til assayed for blastokinin.

nmunoassays. Progesterone was
by specific radioimmunoassay as
y described in our laboratory (10).
highly specific antibody[4] (diluted
n 1:400 normal rabbit serum in
hosphate-buffered saline, pH 7.1)
ɛd to tubes containing dried ben-
ane (1:2) extracts of serum or stan-
antities of progesterone. Then
ɛsterone was added to each tube
tubes were incubated at 4° for
ately 18 hr. Dextran-coated char-
used to separate free from anti-
nd progesterone. Melengestrol
MGA) was tested for its ability to
nding of [^{3}H]progesterone to the
At a concentration of 1 μg per
ɛ cross-reactivity was less than
hus it was not detectable in blood
rom MGA-treated rabbits.

iol was assayed by specific radioim-
ıy using an antibody prepared
stradiol-6-oxime conjugaed to hu-
ım albumin. Validation data for
/ have been reported (11). The
ɛ was similar to that for progester-
ɛpt free and antibody-bound estra-
ɛ separated by addition of sheep
it γ-globulin.

ɛinin was assayed according to the
ɛ of Mayol and Longenecker (12).
blastokinin was iodinated at room
ure by the chloramine T method
incubated with a blastokinin anti-
he presence of unlabeled standard
concentrations of reference blastokinin[5] or with uterine flushing from treated rabbits. A parallel dose–response curve of Day 6 pseudopregnant rabbit uterine extracts with the standard reference preparation was obtained. There was no cross-reactivity of the antibody with rabbit serum samples. To avoid interassay variation, all uterine fluid samples from an experiment were included within a single assay.

Embryo Transfers. Embryos, recovered 72 hr after induction of pregnancy in superovulated donors (9), were transferred to recipients pretreated with MGA or corn oil as described above. Transfers were made at an equivalent stage of pseudopregnancy. Donor oviducts and uteri were flushed with Krebs–Ringer bicarbonate and ova were collected into watch glasses (9). After examination for evidence of cleavage, ova were placed in medium supplemented (1.5 mg of protein/400-μl transfer volume) with either purified blastokinin as prepared by the method of Mayol and Longenecker (12) or rabbit blood serum albumin. The transfer procedure was essentially that described by Staples (13). Each animal served as its own control by receiving a minimum of four embryos per horn; one horn supplemented with blastokinin, the other with serum albumin. Results were recorded as the number of implantation sites on Day 11 of pregnancy per number of ova transferred.

Results. No differences in serum progesterone or estradiol concentrations were detected between rabbits given MGA or corn oil ($P > 0.10$; Figs. 1 and 2). Estradiol fluctuated between 4 and 18 pg/ml prior to and during MGA or corn oil treatment and peaked around 16 pg/ml on the second day of pseudopregnancy. Progesterone was low (<1 ng/ml) prior to pseudopregnancy and peaked on the sixth and seventh days of pseudopregnancy (the last days samples were taken). Similar numbers of large (>2-mm) follicles, hemorragic follicles, and corpora lutea were found on the ovaries of does from each treatment group (Table I).

The effect of corn oil or MGA administra-

bbit antiprogesterone prepared against 6β-
gesterone conjugated to bovine serum al-
supplied by Dr. G. D. Niswender, Colorado
rsity.

[5] Purified blastokinin, guinea-pig anti-rabbit blastokinin, and blastokinin reference preparation were supplied by Dr. R. F. Mayol.

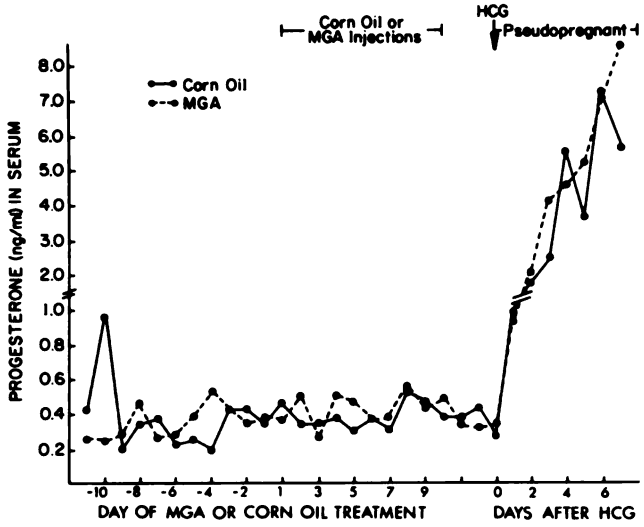


FIG. 1. Progesterone in blood samples collected prior to and during corn oil or MGA treatment and during the subsequent period of induced pseudopregnancy.

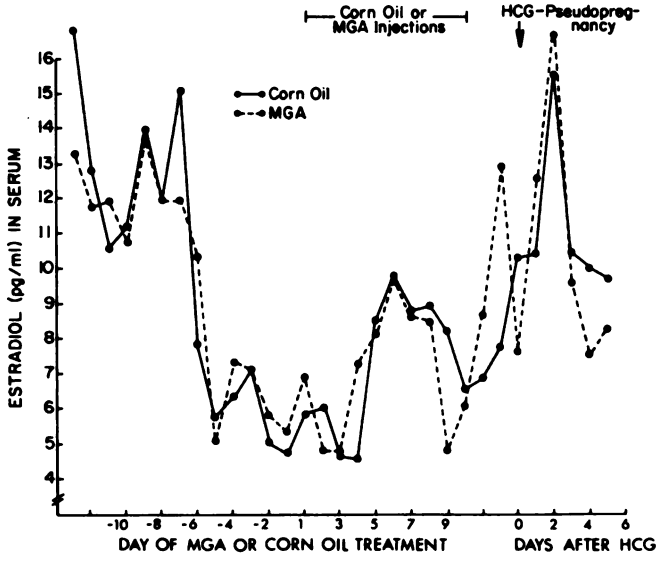


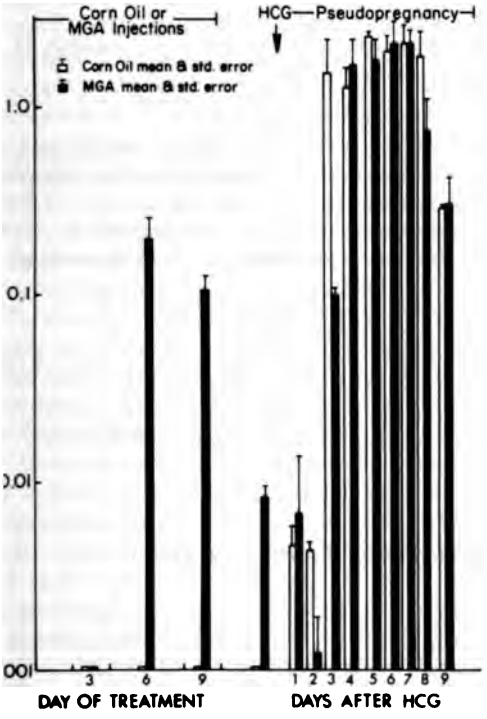
FIG. 2. Estradiol in blood samples collected prior to and during corn oil or MGA treatment and during the subsequent period of induced pseudopregnancy.

tion on blastokinin production during treatment and subsequent pseudopregnancy is shown in Fig. 3. Does given corn oil averaged 0.0004 mg of blastokinin/ml of uterine flushing prior to pseudopregnancy. On Day 3 of pseudopregnancy following corn oil treatment, blastokinin concentrations increased to 1.5 mg/ml, continued to rise until Day 5, plateaued until Day 8 (1.94–2.39 mg/ml), and then fell to 0.29 mg/ml on Day 9. Rabbits given MGA had blastokinin concentrations of 0.006, 0.202, and 0.108 mg/ml on Days 3, 6, and 9 of MGA treatment, respectively ($P < 0.01$). By Day 3 of pseudopregnancy, MGA-treated animals had only 0.1 mg/ml ($P < 0.01$) of blastokinin in the uterine fluid. This concentration increased to 1.6 mg/ml on Day 4, plateaued by Day 7 (1.75–2.23 mg/ml), and then decreased to 0.75 and 0.31 mg/ml

ABLE I. NUMBER OF OVARIAN STRUCTURES ON DAY 7 OF PSEUDOPREGNANCY IN RABBITS PRETREATED WITH CORN OIL OR MELENGESTROL ACETATE (MGA).

	Treatment	
varian structure	Corn oil	MGA
pora lutea	10.3 ± 1.4[a]	11.2 ± 1.5
moragic follicles	2.5 ± 0.3	3.3 ± 0.4
e follicles[b]	8.3 ± 2.0	10.7 ± 1.9

Mean ± standard error of mean.
Follicles >2 mm in diameter.



IG. 3. Blastokinin in uterine flushings collected ng MGA or corn oil treatment and during the sequent period of induced pseudopregnancy.

Days 8 and 9, respectively. The pattern blastokinin secretion during MGA treatnt, although of lesser magnitude, was hly correlated with secretion during udopregnancy ($r = 0.89$). Again follicle nbers did not differ between groups.

Blastokinin-supplemented medium was able to improve implantation rates in s pretreated with MGA when compared h medium containing rabbit serum albu- (4.3 vs 0%). However, when protein- plemented medium was used to transfer embryos to corn oil-treated controls, addition of blastokinin enhanced ($P \simeq 0.1$) survival compared with serum albumin; implantation rates were 32.6 and 23.1%, respectively (Table II). Six of eight does receiving each treatment were pregnant at autopsy on Day 11.

Discussion. These studies support contentions that progestin administration reduces embryo survival by altering the uterine environment (9). The concentration of blastokinin in uterine fluid was reduced on Day 3 of pseudopregnancy in MGA-treated rabbits at a time when fertilized ova first reach the uterus. Lower blastokinin concentrations on Day 3 of pseudopregnancy following MGA were probably due to refractoriness to progesterone secreted by the rabbits' ovaries. This concept is supported by the fact that blastokinin secretion declined in the face of continued progresterone administration to ovariectomized rabbits (4). Since blastokinin concentration was elevated during MGA treatment and began to decline before MGA treatment ended (Fig. 3), the refractory period was evident even before pseudopregnancy was induced.

Because our radioimmunoassay for progesterone could not detect MGA in serum, we could not determine the amount of progestational activity to which MGA-treated animals were exposed during and subsequent to treatment. Runic *et al.* (14) suggest that progestational compounds require longer to be cleared from the system than the natural hormone, and this could account for the delayed rise in blastokinin during the period of pseudopregnancy following MGA treatment.

TABLE II. IMPLANTATION RATE ON DAY 11 OF PREGNANCY OF 72-hr EMBRYOS TRANSFERRED TO MGA- OR CORN OIL-TREATED RECIPIENTS IN MEDIA SUPPLEMENTED WITH RABBIT BLOOD SERUM ALBUMIN OR BLASTOKININ.

Recipient pretreatment	Media supplement[a]	
	Rabbit serum albumin	Blastokinin
Corn oil	9/39[b] (23.1%)	14/43 (32.6%)
MGA	0/43 (0%)	2/47 (4.3%)

[a] 1.5 mg of protein/400-μl transfer volume.
[b] Number of embryos present on Day 11/number of 72-hr embryos transferred.

These results also lend credence to reports (15, 16) that blastokinin stimulates growth and development of early blastocysts *in vitro*. Blastokinin was able to enhance implantation in control animals, but was unable to overcome the infertility effects of progestin administration.

Summary. Progesterone and estradiol were measured in blood and blastokinin was assayed in uterine flushings collected from rabbits prior to and during treatment with melengestrol acetate (MGA) or corn oil and during the subsequent period of induced pseudopregnancy. Blastokinin was elevated during MGA treatment, but was transiently suppressed on Day 3 of pseudopregnancy after MGA when levels in corn oil-treated rabbits were 10-fold higher than those in rabbits given MGA. Estradiol and progesterone were not different between rabbits given the two treatments. Addition of purified blastokinin to embryo transfer media increased implantation rates in corn oil-treated recipients, but not in recipients pretreated with MGA.

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ıtion of Vesicular Stomatitis Virus Facilitated in Nonpermissive Cells by Early Functions of Shope Fibroma Virus (40026)

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ett and Walker reported several go (1) that replication of vesicular tis virus (VSV), an RNA virus, is ly facilitated in serially cultured do- rabbit kidney (DRK_3) cells (2) per- / infected with a noncytocidal strain e fibroma virus (SFV), a DNA virus. oserved that superinfection of DRK_3 ; with VSV 3–5 days after treatment fectious SFV resulted in yields of 0,000-fold greater than in parallel ; challenged by VSV alone. Re- we reported (3) that this dramatic ion of VSV also occurs when DRK_3 ; are inoculated simultaneously with ruses, suggesting that contempora- replication of SFV, if it occurs in ells, does not interfere with the rep- of VSV. In the same study we found raviolet irradiation of SFV abolishes ity to induce facilitation, indicating unctional SFV genome is necessary. therefore of interest in the present) determine (i) whether SFV does in olicate in the presence of VSV and ther the postulated viral gene func- quired for facilitation are expressed late in the replication of SFV, i.e., or after DNA synthesis. In addition, SV by itself appears unable to repli- DRK_3 cells (1, 3), cultures chal- with VSV in the absence of SFV xamined for the induction of viral hic effects (CPE) to determine r the cells are completely nonpermis- whether they become abortively in-

rials and methods. Cloned DRK_3 iginally isolated by Dr. H. C. Hinze University of Wisconsin, Madison, own as monolayers in Eagle's mini- sential medium (MEM) supple- with 10% heat-inactivated fetal bo- rum, 0.23% sodium bicarbonate, ts/ml of penicillin, and 100 μg/ml of streptomycin. Vero cells provided by W. Wong, State Hygienic Laboratory, University of Iowa, Iowa City, were grown in monolayers in the same medium with only 5% serum. For maintenance of both kinds of cells following viral infection, the concentration of serum was reduced to 2.5%. Experimental cultures were prepared by seeding screw-capped Leighton tubes (11 × 38-mm window) with 1×10^5 DRK_3 or Vero cells in 1 ml of the appropriate growth MEM. After incubation at 37° in 5% CO_2 for 2 days, the cultures routinely contained $2\text{–}4 \times 10^5$ actively growing cells and were used for experimentation.

The Patuxent strain of SFV, noncytolytic in DRK cultures (4), was also obtained from Dr. Hinze. Stocks of cloned virus were grown at 34° and harvested as described previously (2). The Indiana strain of VSV was provided by Dr. J. E. Rodriguez, University of Iowa, Iowa City. Stocks of cloned VSV were prepared by inoculation of Vero cultures with about 1×10^{-5} infectious units/cell. This low multiplicity of infection was used to avoid production of defective T forms of the virus (5). When VSV-specific cytopathic effects (CPE) characterized by rounded, detached cells were apparent in 100% of the cells, generally 36 hr after incubation at 37°, the culture fluids were clarified by centrifugation at 800*g*, pooled, and stored at −55°.

Infectious SFV and VSV present together in doubly infected DRK_3 cultures were quantitated by a differential assay that exploited a marked difference in the host range of the two viruses. Whereas diluted SFV caused foci of aggregated cells in DRK_3 monolayers, as previously described (6), diluted VSV had no demonstrable affect. In contrast, VSV rapidly produced cytolytic plaques in monolayers of Vero cells, while SFV was unable to form either plaques or foci. Cultures to be assayed for both viruses

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were frozen and thawed to detach the cells and then subjected to sonification in a Branson Sonifer (Cole-Parmer Instruments & Equipment Co.) for cellular disruption. After serial dilution of the resultant culture fluids in cold Tris-buffered saline (pH 7.4), 0.1-ml amounts were inoculated onto confluent monolayers of both DRK_3 and Vero cells in 35-mm plastic petri dishes. Following viral adsorption for 2 hr at 37°, 2 ml of MEM containing 0.5% methylcellulose (Matheson, Coleman, and Bell), 5% heat-inactivated fetal bovine serum, and the usual concentrations of sodium bicarbonate and antibiotics was added to each monolayer. The Vero cultures were then incubated at 37° and the DRK_3 cultures at 34°. After 24 hr the inoculated Vero monolayers were treated with 10% formalin containing 0.05% methylene blue; the DRK_3 monolayers were similarily treated after incubation for 5 days. With the aid of a binocular dissecting microscope, after the fixed and stained cultures were rinsed and air-dried, foci of aggregated SFV-infected DRK_3 cells and plaques of cytopathically affected VSV-infected Vero cells were readily counted. Based on experiments in which known amounts of SFV and VSV were mixed, diluted, and assayed in this manner, the presence of each virus had no demonstrable affect on quantitation of the other in its respective permissive host.

To quantitate amounts of DNA synthesized, the material in cultures labeled with 10 μCi of [^{3}H]thymidine ([*methyl*-^{3}H]thymidine; 58 Ci/mmol; Schwarz/Mann) was first digested and then subjected to either equilibrium centrifugation in cesium chloride or direct acid-precipitation. Each culture to be assayed was thawed and treated for 3 hr at 37° with a mixture containing a final concentration of 0.5% Sarkosyl NL30 (Geigy Chemical Corp.), 0.02 *M* disodium (ethylenedinitrilo)tetraacetate (EDTA; pH 7.0), and 0.1% Pronase (Calbiochem, B grade). Sarkosyl and Pronase were prepared in 0.015 *M* sodium chloride and 0.0015 *M* sodium citrate (0.1× SSC, pH 7.3). For equilibrium centrifugation, 200 μl samples from each digested culture were mixed in separate nitrocellulose tubes (0.5 × 2.5 in.) with 3.8 ml of 0.1× SSC-buffered cesium chloride (Kerr-McGee Chemical Corp., ρ = 1.733 g/cm^3) and centrifuged at 30,000 rpm for 60 hr in a refrigerated Beckman L centrifuge using a Type 40 fixed-angle rotor. Ten-drop fractions were dripped from the bottom of the gradients and collected on filter paper disks (Whatman 3M, 25 mm). After collection, the filters were placed in 5% trichloroacetic acid at room temperature to precipitate acid-insoluble material. The filters were then dehydrated in ethanol and acetone, air-dried, and placed in 10 ml of Omnifluor (New England Nuclear Corp.) to measure ^{3}H by liquid scintillation. Gradient densities were determined by measuring the refractive indexes of periodic fractions in a Bausch and Lomb refractometer. For direct precipitation, a 0.5-ml sample from each digested culture was transferred to a glass tube containing 0.5 ml of 10% trichloroacetic acid. Precipitates were allowed to form at room temperature for 1 hr. The entire content of each tube was then transferred quantitatively to separate glass-fiber disks (Whatman GF/C, 24 mm) positioned in a Millipore filtration manifold. After washing the precipitates with 5% trichloroacetic acid and 95% ethanol, the disks were removed, air-dried, placed in 10 ml of Omnifluor, and analyzed by liquid scintillation for total ^{3}H counts per minute.

Results. To determine whether SFV replicates in the presence of VSV, actively growing DRK_3 cells were inoculated with 0.2 ml of a mixture containing SFV plus VSV diluted in MEM without serum to give respective multiplicities of 10 and 20 infectious units/cell. Control cultures were inoculated with each virus separately at similar multiplicities. Following viral adsorption at 37° for 2 hr, the inocula were removed, each culture was rinsed once with 5 ml of Tris-buffered saline, and 1 ml of maintenance MEM was then added. At 3 and 30 hr after inoculation, replicate cultures were placed at −55° and assayed later for infectious SFV and VSV by the differential method described above.

Data in Table I show that both viruses replicated in the dual-inoculated cultures. At 3 hr, the amount of residual input virus was routinely only 10^3–10^4 SFV focus-form-

TABLE I. REPLICATION OF SFV AND VSV IN DUALLY INOCULATED DRK_3 CELLS.

Inoculum[a]	Yields of infectious virus[b]	
	SFV FFU/ culture	VSV PFU/ culture
FV + VSV	1.2×10^6	2.5×10^6
SFV	1.3×10^6	
VSV		6.4×10^3

Cultures were inoculated with a mixture of SFV VSV or with each virus separately at multiplicities SFV FFU/cell and 20 VSV PFU/cell.
Infectious virus was assayed 30 hr after infection.

units (FFU)/culture and 10^4-10^5 VSV ue-forming units (PFU)/culture (data shown). Since the amount of infectious produced by 30 hr in the doubly in-d cultures was essentially the same as ie cultures infected with SFV alone, the emporaneous replication of VSV caused nterference or enhancement of SFV. In rast, while production of SFV was in-ndent of VSV, replication of VSV ap-ed to be completely dependent on the ence of SFV.

o determine whether facilitation of VSV ese dually infected DRK_3 cultures re-es early or late functions of the SFV me, replication of VSV was examined ie presence of increased concentrations ydroxyurea to inhibit DNA synthesis. y poxviral genes are expressed before hesis of the viral DNA (7). Cultures of ely growing cells were treated with dif-nt amounts of the inhibitor at 37° for 4 nd then inoculated with virus. For inoc-on, immediately after removal of the ium 0.1 ml of 2× concentrated hydrox-a appropriately diluted in MEM with-serum was added to each culture. To was added 0.1 ml of a mixture contain-oth SFV and VSV diluted in the same M to give the usual final multiplicities nfection. Control cultures were inocu-l in the same manner with either SFV /SV alone. After viral adsorption the ires were washed and nourished with M as described in the previous experi-t. Appropriate concentrations of hy-yurea were included in the medium. cultures were then divided into two ps and given an additional 0.1 ml of medium plus or minus 10 μCi of [^{3}H]thymidine. Incubation was continued at 37°. At 3 and 30 hr after infection, cultures not treated with radioisotope were placed at −55° to be assayed later for infectious SFV and VSV. Cultures labeled continuously with [^{3}H]thymidine until 30 hr after infection were also frozen at −55° and then assayed later by equilibrium centrifugation.

Results in Table II show that SFV was able to facilitate VSV replication in the presence of hydroxyurea. At all of the concentrations of inhibitor tested, by 30 hr after infection, yields of VSV in the dually infected cultures were more than 10,000-fold greater than in controls inoculated with VSV alone. These yields were comparable to those produced in the absence of the drug. Interestingly, in repeated experiments, yields of VSV were somewhat lower at 100 m*M* hydroxyurea than at either 10 or 50 m*M*. Because the presence of hydroxyurea did not permit VSV to replicate in cultures inoculated with VSV alone, the facilitation of VSV observed in the doubly infected cultures was caused by SFV rather than by some effect induced by the drug. When these various cultures were assayed for infectious SFV, including those inoculated with SFV alone, production of virus was found to have been inhibited by greater than 99% at all three concentrations of hydroxyurea (data not shown).

The extent of inhibition of DNA synthesis by hydroxyurea in the dually infected cultures is presented in Fig. 1. Rather than for successive short pulses, [^{3}H]thymidine was

TABLE II. FACILITATION OF VSV IN DRK_3 CELLS BY SFV IN THE PRESENCE OF HYDROXYUREA.

Inoculum[a]	Hydroxyurea[b] (m*M*)	Yield of VSV[c] (PFU/culture)
SFV + VSV	0	7.5×10^8
SFV + VSV	10	3.3×10^9
SFV + VSV	50	3.8×10^9
SFV + VSV	100	1.0×10^8
VSV	0	7.8×10^3
VSV	100	1.0×10^3

[a] Cultures were inoculated with a mixture of SFV and VSV or with VSV alone at multiplicities of 10 SFV FFU/cell and 20 VSV PFU/cell.
[b] The drug was present 4 hr before, as well as throughout the entire 30 hr period of infection.
[c] Infectious VSV was assayed 30 hr after infection.

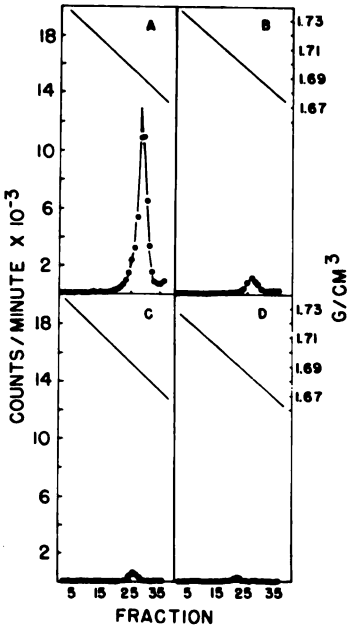
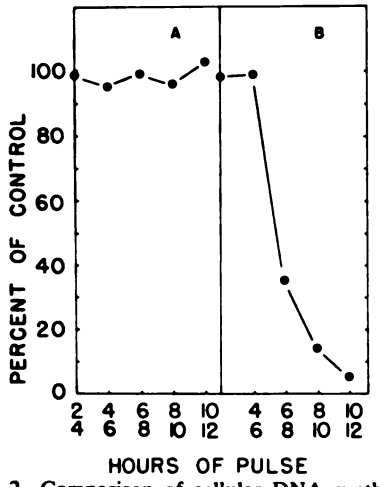


FIG. 1. Analysis of DNA synthesis in cultures of DRK_3 cells inoculated simultaneously with SFV and VSV in the absence (A) or presence of hydroxyurea at concentrations of 10 m*M* (B), 50 m*M* (C), or 100 m*M* (D). Nonconfluent DRK_3 cultures were treated with the various concentrations of hydroxyurea for 4 hr at 37° and then inoculated with a mixture containing 10 SFV FFU/cell, 20 VSV PFU/cell, and the appropriate amounts of the inhibitor. After 2 hr at 37°, 10 μCi of [^{3}H]thymidine was added and remained continuously until 30 hr postinfection. Samples were then digested with Pronase and Sarkosyl in the presence of EDTA, subjected to isopycnic centrifugation in cesium chloride, and assayed for acid-precipitable radioactivity.

present continuously for an extended period of time to detect low levels of DNA synthesis. Because the guanidine-cytosine content of SFV DNA (8) is similar to that of rabbit cell DNA (9), the assay method used was not expected to distinguish between viral and cellular DNA. Equilibrium centrifugation was used as a means to identify the ^{3}H-labeled material as DNA. In the absence of hydroxyurea there was a single sharp peak of ^{3}H-labeled DNA at the characteristic buoyant density of 1.69 g/cm^3. Despite inhibition of infectious SFV production by greater than 99%, continuous labeling for 28 hr revealed the synthesis of small amounts of DNA at all of the concentrations of hydroxyurea tested. Calculations based on the total counts per minute in each peak showed inhibitions of 89, 95, and 9 respective drug concentrations of 1 and 100 m*M*. Similar results were fo repeated experiments. Although these amounts of DNA were synthesized unlikely that they were necessary fo to facilitate VSV. As shown in Tab except for the somewhat decreased y infectious VSV at 100 m*M* hydrox probably caused by drug cytotoxicity, was apparent in parallel noninfected c cultures, the marked inhibition of synthesis by hydroxyurea did not affe extent of facilitation. In fact, inhibito centrations of 10 and 50 m*M* seem enhance replication of the VSV.

To determine whether DRK_3 cel nonpermissive for VSV alone, or w they are abortively infected, cultures t with the virus were examined for (i) tion of cellular DNA synthesis and duction of viral CPE. As a positive c for this experiment, permissive Ver were examined in parallel with the cells. Actively growing cultures cont comparable numbers of each kind were inoculated with 0.2 ml of VSV c in maintenance MEM to give a multi of 20 PFU/cell. Control cultures mock-infected with the MEM only. A hr at 37°, 1 ml of the same mediu added to each culture without remo the inoculum. At various times after ulation replicate cultures were pulse-l for 2 hr by the addition of 10 μ [^{3}H]thymidine. Following each puls cultures were frozen at −55° until a for the amount of [^{3}H]thymidine in rated into acid-precipitable material, scribed above. Total ^{3}H counts per r present in the virus-treated cultures then compared with those present mock-infected controls.

As shown in Fig. 2A, in DRK_3 c VSV was unable to inhibit incorporat [^{3}H]thymidine into material presum be cellular DNA. Even after 24 hr not shown) no inhibition was obs Contrastingly, synthesis of cellular was markedly inhibited by VSV in th missive Vero cultures. By 10 hr after ulation, incorporation of label into t rus-treated cultures was about 95%



IG. 2. Comparison of cellular DNA synthesis in -treated and untreated DRK_3 cells (A) and Vero (B). Nonconfluent cultures of both kinds were ılated with maintenance MEM or the same me- containing 20 VSV PFU/cell. At the indicated s, 10 μCi of [^{3}H]thymidine was added to replicate res for a pulse period of 2 hr at 37°. Each point sents the total amount of acid-precipitable ^{3}H-ed material in the virus-treated cultures relative at present in the mock-treated controls pulsed at ame time.

ı in the controls. Microscopic examina- of the various cultures showed similar erences. At 24 hr, whereas no detectable / CPE was seen in the virus-treated K_3 cultures, 100% of the Vero cells ıifested the rounded appearance charac- ıtically induced by VSV infection. In nissive cells inoculated with VSV alone, bition of cell macromolecular synthesis ears to be associated with the develop- t of CPE (10, 11). Since induction of at low input multiplicities requires hesis of VSV messenger RNA (12, 13) scribed by the virion-associated tran- ptase (14), failure of VSV to inhibit A synthesis or induce CPE in DRK_3 indicates that these cells are completely permissive for this virus.

iscussion. The results of this investiga- demonstrate that some as yet unknown ıt early in the replicative cycle of SFV, r to synthesis of its DNA, facilitates the ication of VSV in DRK_3 cells. This ing is similar to that of Thacore and ngner (15), who reported that replica- of VSV in rabbit cornea (RC-60) cells is markedly facilitated by vaccinia virus, a cytolytic poxvirus related to SFV, in the absence of viral DNA synthesis. Thus, it appears that facilitation of VSV in certain restrictive cells is a property common to different poxviruses.

In addition to supporting the contention that poxviruses characteristically facilitate VSV in some cells, the present study also shows that contemporaneous replication of VSV in such cells has no affect on replication of the facilitating poxvirus. In DRK_3 cells, at least, SFV replicated to the same extent whether or not VSV was replicating at the same time. Apparently, there is little or no competition between the two viruses during synthesis and maturation of progeny virions.

While similarities exist between facilitation of VSV by poxviruses in DRK_3 and RC-60 cells, both require early poxviral functions, a significant difference is also apparent. Replication of VSV alone in RC-60 cells reportedly is abortive; at multiplicities of 5–10 PFU/cell, viral CPE occurs, some viral RNA is synthesized, and small amounts of viral cores are detectable (15). In contrast, the present study indicates that DRK_3 cells are nonpermissive for VSV. At a comparable multiplicity of 20 PFU/cell, no virus-induced inhibition of cellular DNA synthesis occurred, and viral CPE did not develop. Thus, interaction between VSV and SFV in these cells appears to be unique. Since in permissive cells infected by VSV alone at these low input multiplicities induction of CPE seems to require primary transcription of the viral genome (12), the failure of VSV to cause CPE in DRK_3 cells suggests that viral replication in these cells, unlike RC-60 cells, is blocked before this initial synthetic event occurs. One very early step in VSV replication that may be blocked is viral adsorption. Since it is known that SFV alters the plasma membrane of infected DRK_3 cells (16), it is conceivable that SFV modifies the cell surface to provide receptors which then allow VSV to be adsorbed. This may explain why the presence of VSV does not affect focus formation by SFV in our differential assay of the two viruses. If VSV in a mixed inoculum is unable to attach to monolayers of DRK_3 cells during

the period for viral adsorption, addition of the overlay medium would likely prevent it from later infecting the foci of SFV-infected, permissive cells. Whether infection by SFV does in fact facilitate VSV adsorption to these cells, however, will require further investigation, especially since in their original study Padgett and Walker (1) concluded that VSV is adsorbed in the absence of SFV.

Summary. In serially cultured domestic rabbit kidney (DRK_3) cells, vesicular stomatitis virus (VSV) alone was unable to replicate, inhibit synthesis of cellular DNA, or induce CPE. When these cells were inoculated simultaneously with VSV plus Shope fibroma virus (SFV), both viruses replicated without apparent interference. This faciliation of VSV by SFV also occurred when these dually inoculated cells were treated with hydroxyurea at concentrations which inhibited production of infectious SFV by greater than 99% and synthesis of viral and cellular DNA with a buoyant density in cesium chloride of 1.69 g/cm^3 by greater than 90%. These results indicate that an early event in the replication of SFV, before viral DNA synthesis, converts DRK_3 cells from a nonpermissive state, in which VSV alone is not even able to induce CPE, to a permissive state that allows complete VSV replication.

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lesterol and Hormone Levels in Sera of Juvenile Female Rhesus Monkeys[1] (40027)

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investigators have reported that ıs and androgens may affect blood ıe lipids (1, 2). Estrogen administra- been shown to increase serum high (α) lipoproteins and decrease low (β) lipoproteins (3, 4). Barr *et al.* noted the inverse relationship be- he ratio of α/β lipoproteins and bility to coronary heart disease. This ship has been given considerable y as newer means of measuring the ıprotein ratio have become available

n *et al.* (9) reported an increase in holesterol levels following oopho- , and Mann and Thorogood (10) ggested that ovarian hormones may premenopausal women from Type rlipoproteinemia.

of the data on the effect of estro- d androgens on serum lipids and eins concern middle-aged men and little is known about these effects life. Since the rhesus monkey is l to have a menstrual cycle and e levels comparable to those in (11), the effects of 17β-estradiol osterone propionate on the plasma els of juvenile female monkeys were

ials and methods. Fifteen 5-month- pubertal female rhesus monkeys *ue mulatta*) weighing 1.1 ± 0.4 kg d a laboratory monkey ration *ad* The diet was supplemented with ıd vitamins. The monkeys were weekly and their stage of sexual ment and evidence of menses re- Five monkeys were given subscapular implants of Silastic capsules containing 10 mg of 17β-estradiol. Five other monkeys were given similar implants containing 15 mg of testosterone propionate. Capsules were prepared according to the method of Karsch *et al.* (12) and were replaced every month over the course of the study.

Blood samples were taken monthly until menarche, which occurred when the body weight of the monkeys reached 3.4 kg. Many of the initial menses were anovulatory.

Blood was drawn from the femoral vein using heparinized syringes. Plasma estradiol and testosterone levels were measured by radioimmunoassay (RIA), the former by the method of England *et al.* (13) and the latter according to Furuyoma *et al.* (14). Plasma cholesterol and triglyceride levels were determined by standard Autoanalyzer methodology as described by Schwartz and Hill (15). The low (β) and high (α) density lipoproteins were separated by dextran sulfate fractionation (16) and the cholesterol content of the fractions was determined (17). The lipoprotein phospholipid content was determined by the method of Kraml (18). Livers, ovaries, and adrenal glands were weighed when the animals were killed. Statistical differences were computed using Student's *t* test.

Results and discussion. There were no significant differences in weight gain between treated and untreated monkeys (Fig. 1). Cholesterol levels of all groups showed a type of seasonal variation which had been seen previously in monkeys (19); however, cholesterol levels were reduced in both groups of hormone-treated monkeys. The estrogen-treated monkeys exhibited the lowest cholesterol levels throughout (Fig. 1). Phospholipid levels were significantly lower in the two hormone-treated groups and were also consistently lower in the

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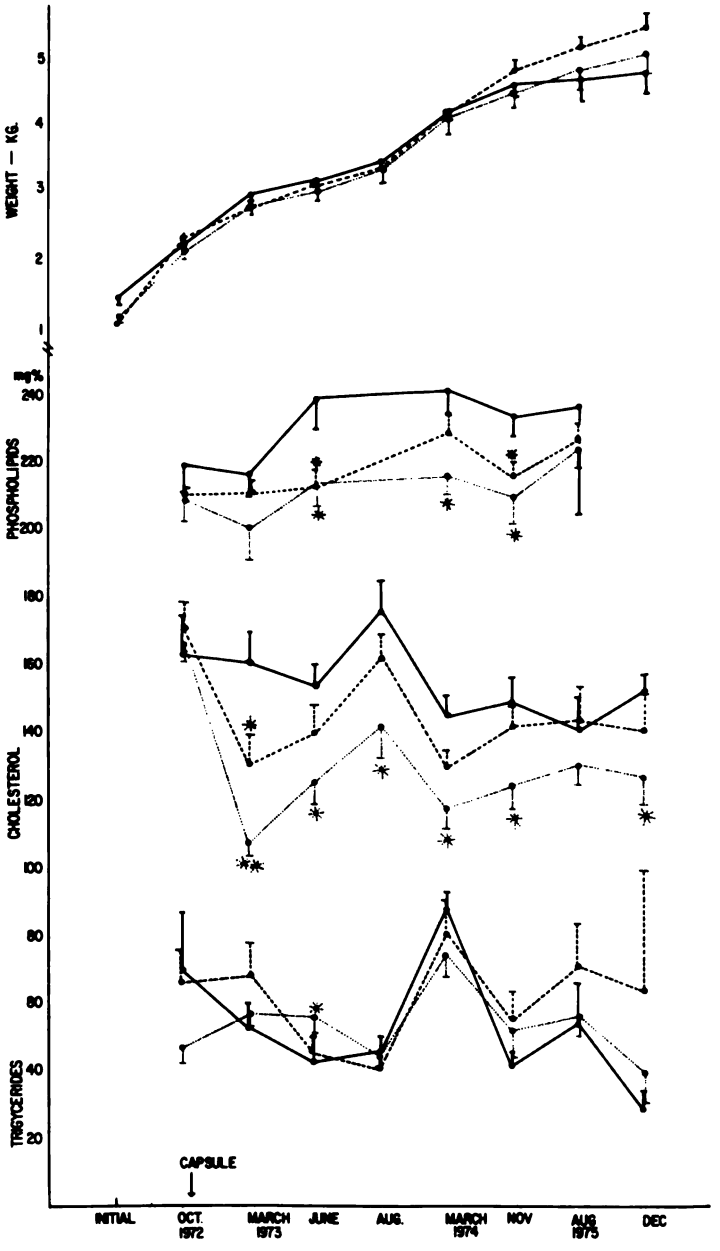

FIG. 1. Changes in body weight and plasma lipids in untreated monkeys (●——●) and in monkeys bearing implants of testosterone proprionate (●- - -●) or 17β-estradiol (O· · · ·O). (*)$P < 0.05$; (**)$P < 0.01$.

estradiol group (Fig. 1). Triglyceride levels were generally comparable for all groups. The plasma cholesterol levels and the α/β lipoprotein cholesterol ratios are shown in Table I. The estradiol group exhibited the lowest levels of plasma cholesterol and of α/β lipoprotein cholesterol ratio.

Implantation of 17β-estradiol or testosterone resulted in significantly elevated levels of the respective hormones (Figs. 2 and 3). Delayed development of sexual characteristics such as sex skin and clitoral hypertrophy were evident in the testosterone-treated monkeys.

TABLE I. TOTAL PLASMA CHOLESTEROL, α LIPOPROTEIN CHOLESTEROL, AND α/β LIPOPROTEIN CHOLESTEROL LEVELS IN MONKEYS AFTER 2 YEARS OF ESTROGEN OR ANDROGEN TREATMENT.

Group	No.	Plasma cholesterol (mg/dl)	α Lipoprotein cholesterol (mg/dl)	α/β Lipoprotein cholesterol ratio
Control	5	152 ± 4.2[a]	57 ± 5.3	0.60 ± 0.07
Testosterone propionate	5	140 ± 11.6	48 ± 2.9	0.55 ± 0.08
17β-Estradiol	5	126 ± 7.9*	40 ± 2.7**	0.49 ± 0.05

[a] Standard error.
* $P < 0.02$ vs control.
** $P < 0.05$ vs control.

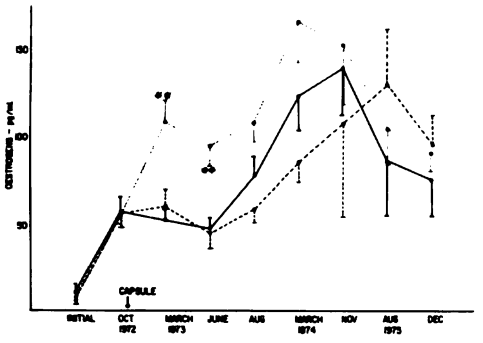

FIG. 2. Plasma estrogen levels in untreated monkeys (●——●) or in monkeys bearing implants of testosterone propronate (●- - -●) and 17β-estradiol (O····O) during puberty. (**)$P < 0.01$.

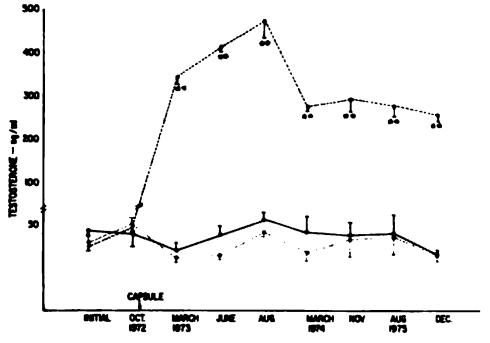

FIG. 3. Plasma testosterone levels in untreated monkeys (●——●) or in monkeys bearing implants of testosterone propionate (●- - -●) and 17β-estradiol (O····O) during puberty. (**)$P < 0.01$.

There were no significant changes in the weights of livers, ovaries, or adrenal glands (Table II).

In man, serum cholesterol increases rapidly after birth and then usually increases progressively with age. An increase in serum cholesterol early in life has also been reported for nonhuman primates (20). In man the lipoprotein level and distribution of lipids between the lipoprotein classes appear to be established prior to puberty (21).

Estradiol levels in the rhesus monkey increase during puberty (22), but few data are available on lipoprotein levels in prepubertal monkeys (23). Methyl testosterone and other testosterone analogs have been reported to lower serum lipids in hyperlipidemic individuals (24), although their effect on the α and β lipoproteins may be diet dependent (25). In this study prolonged elevation of testosterone levels did not effect significant lowering of cholesterol levels. Testosterone propionate treatment did not influence weight gain, although marked signs of virilization were evident.

The lower cholesterol levels observed in monkeys bearing estradiol implants may reflect the effects of raising plasma estrogen levels before puberty to the level found in early follicular and late luteal phases of the menstrual cycle. Estrogen has been reported to transfer cholesterol from serum to liver (26) and to alter catabolism of the very low density lipoproteins (27) and the α lipoproteins (28). Plasma lipids are elevated in oophorectomized women (9) and the risk of coronary heart disease in women increases after menopause (29). The higher levels of plasma estradiol in African girls, as compared to their Caucasian counterparts, has been correlated with a lower risk of coronary heart disease (30).

It would be of interest to determine to what degree serum hormone levels in prepubertal rhesus monkeys can be modified by diet and other environmental factors.

Summary. Juvenile female rhesus monkeys were treated with either 17β-estradiol or testosterone propionate (by implanta-

TABLE II. ORGAN WEIGHTS IN FEMALE JUVENILE RHESUS MONKEYS (g).

	Weight (kg)	Adrenal glands	Ovaries	Liver	Liver as % body wt
Untreated control (5)[a]	4.82 ± 0.29	1.00 ± 0.12	0.35 ± 0.07	91.6 ± 2.3	1.90
17β-Estradiol capsule (5)	5.08 ± 0.26	1.05 ± 0.07	0.42 ± 0.09	89.3 ± 7.5	1.76
Testosterone propionate capsule (5)	5.23 ± 0.22	0.93 ± 0.11	0.39 ± 0.06	99.2 ± 12.7	1.90

[a] Number of animals given in parentheses.

tion) and the hormonal levels were kept elevated to puberty. There was no effect on weight gain in either group. Estradiol lowered plasma cholesterol and phospholipid levels significantly. Neither treatment had a significant effect on plasma α/β lipoprotein cholesterol ratios or on the weights of liver, ovaries, or adrenals.

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halamic Somatostatin and LH-RH after Hypophysectomy, in Hyper- or Hypo-Thyroidism, and during Anesthesia in Rats (40028)

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ants of growth hormone (GH) in the :dian eminence decrease pituitary and GH content, suggesting a nega- :dback effect of GH at the hypotha- :vel (1). Demonstration of the pres- : TSH in the hypothalamus also im- ıat TSH might affect hypothalamic (2). Recently Oliver *et al.* (3) re- that hypophysial portal blood not ows from median eminence to the y gland but, as well, there is retro- :irculation via the neurohypophysis primary capillary plexus. Further, plasma contains high concentrations ı neurohypophysial and adenohypo- hormones. These results favor a ıat the pituitary hormones might ex- irect feedback control on the hypo- ropic hormones of the hypothala-)n the other hand, thyroidectomy in the degranulation of the pituitary ıil cells in rats (4), but also decreases y GH content (5), and impairs GH)n (6). The mechanism by which T_4 influence GH synthesis in the pitui- still unknown, but these thyroid ıes appear to exert a direct effect on ıitary gland (7).

anesthetics are known to change e of secretion of various pituitary ıes, probably by an effect mediated hypothalamus. The administration ium pentobarbital (Nembutal) into ıcks the preovulatory surge of gonad- and ovulation (8). Nembutal in- serum GH and prolactin levels (9, hereas urethane reduces GH and in (11).

recent studies using antiserum to somatostatin indicated that this tetradecapeptide is involved in a regulatory mechanism of GH and TSH secretion in rats (12).

The following questions could be raised: 1. If a short feedback regulatory mechanism takes place for GH and TSH secretion, does the removal of GH and TSH by hypophysectomy affect the hypothalamic somatostatin? 2. Does the absence or the excess of circulating thyroid hormone affect the hypothalamic somatostatin? 3. Do Nembutal and urethane modify somatostatin content of the hypothalamus? Our studies were designed to answer some of these questions.

Materials and methods. Experimental procedures. Young adult male rats of CD strain from Charles River were used throughout the experiments. They were maintained at a constant temperature (24°) and illumination (lights on 0500–1900 hr) and were given free access to tap water and Purina laboratory chow.

In Exp. I, the rats were hypophysectomized when they were 35-days old and sacrificed by decapitation 24 days or 2.5 months after the operation. Intact age-matched rats served as controls.

In Exp. II, 70-day-old male rats, weighing 300–350 g, were divided into the following four groups: Group 1, intact control rats; Group 2, thyroidectomized rats. These two groups were injected with 0.2 ml of 0.9% saline subcutaneously (sc). Group 3, thyroidectomized rats were injected with 0.1 μg of L-thyroxine (T_4) sc; and Group 4, normal rats were injected with 10 μg of T_4 sc. All groups were injected every 2 days for 10 days.

In Exp. III, the rats were injected with 4.5 mg Nembutal/100 g body weight (bw) intraperitoneally (ip) or 150 mg urethane/100 g bw ip. They were sacrificed by decap-

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itation 5, 10, 30 min to 1 or 2 hr after the injection of the anesthetic. The blood was allowed to clot; the serum was separated by centrifugation and kept at −20° until assayed for GH and TSH.

Extracts of the hypothalami of rats. Immediately after decapitation, the hypothalamic tissue from the posterior margin of the optic chiasma to the mammillary body and laterally to the lateral boundary of the tuber cinereum and dorsally approximately 1 mm from the ventral surface, was dissected and immediately frozen. The hypothalamic tissue was then homogenized in 1 ml of ice-cold 2 *N* acetic acid, heated for 5 min in boiling water, chilled, and centrifuged at 3000 rpm for 15 min at 4°. Seven-tenths ml of the supernatant was lyophilized. The residue was dissolved in a diluent for radioimmunoassay (RIA) shortly before the assay. The inhibition curves of the hypothalamic extracts in the RIA for somatostatin and LH-RH were parallel to those of synthetic somatostatin and LH-RH, respectively. The results were expressed as ng of somatostatin or LH-RH per hypothalamus (ng/SEM) and per 100 g bw.

Radioimmunoassay. LH-RH was measured by RIA as described by Arimura *et al.* (12) using rabbit antiserum #743 at a final dilution of 1:28000. The sensitivity was 1 pg per tube. The antigenic determinant of this antiserum resided in the amino acid sequence from position 3 to 9. The RIA for LH-RH was quite specific and did not exhibit any significant cross-reaction with other hypothalamic and pituitary hormones.

Somatostatin was also determined by RIA as described previously (14) using rabbit antiserum to cyclic somatostatin #101 at a final concentration of 1:28000. *N*-Tyr-Somatostatin, a pentadecapeptide prepared by Dr. C.A. Meyers, was iodinated by ^{125}I using lactoperoxidase, purified on a CMC column, and used as a tracer. The standard curves obtained with ^{125}I-*N*-Tyr-Somatostatin and ^{125}I-Tyr^1-Somatostatin were exactly the same. ^{125}I-*N*-Tyr-Somatostatin was more stable than ^{125}I-Tyr^1-Somatostatin. After storage for 1.5 months at −50°, the former could be used for RIA without repurification. The cyclic somatostatin was used as the reference standard. The diluent for RIA was 0.25% human serum albumin (HSA)/0.1% gelatin/0.14 *M* NaCl/0.025 *M* EDTA/0.01 *M* phosphate buffer pH 7.5. The sensitivity of the assay was 1 pg per tube.

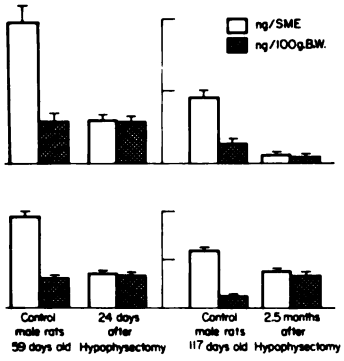
The serum TSH and GH levels were determined by radioimmunoassay (RIA), using the rat pituitary hormone RIA kits provided by NIAMDD. The data were analyzed using a computer program described by Duddleson (14).

Statistical analysis. The mean concentrations of somatostatin or LH-RH in the hypothalamus and in the various treatment groups were compared by using factorial analysis of variance, and the effect of each treatment was tested for a significant level. Mean serum GH, TSH, prolactin, and LH levels in various groups were also compared with one another by Duncan's new multiple range test (16).

Results. The effect of hypophysectomy on the hypothalamic somatostatin and LH-RH content. The mean hypothalamic somatostatin content in 58-day-old rats which were hypophysectomized 24 days previously was 15.1 ± 1.78 (SE) ng, whereas that of the age-matched intact rats was 49 ± 4.9 ng (Fig. 1). The mean somatostatin content of 117-day-old rats which were hypophysectomized 79 days before was 2.2 ± 0.5 ng, and that of the age-matched intact rats was 24.5 ± 2.4 ng.

Two by two factorial analysis of variance indicated that both hypophysectomy and aging affected hypothalamic somatostatin content, and that there was a significant interaction between these two factors. The somatostatin content in 117-day-old, hypophysectomized rats was significantly smaller than in any of the other groups. The hypothalamic somatostatin content of the older rats (117 days old) was significantly lower than that of the younger animals (58 days old) in both intact and hypophysectomized animals.

When the hypothalamic somatostatin was expressed as ng/100 g bw, the content in hypophysectomized 58-day-old rats was not significantly different from that in the age-matched animals, whereas the content in the hypophysectomized, 117-day-old rats



he effect of hypophysectomy on the hy-
omatostatin and LH-RH contents. The
indicate ± SEM. The hypothalamic so-
nd LH-RH contents (ng/SME) 24 days
ıths after hypophysectomy were signifi-
than those of their respective control
ents of the hypothalamic somatostatin and
xpressed in ng/SME and ng/100 g BW) in
ats were significantly lower than those in
ats ($P < 0.05$). The hypothalamic so-
ontent (ng/100 g bw) 2.5 months after
my was significantly lower than that in
ed intact rats ($P < 0.05$).

icantly smaller than that of the
ed intact rats.

ın hypothalamic LH-RH contents
/sectomized 58- and 117-day-old
similar; 3.5 ± 0.3 ng/SME and
ng/SME, respectively. On the
., the mean hypothalamic LH-RH
the age-matched intact rats were
ng/SME and 6.0 ± 0.8 ng/SME,
y, the difference being significant
As in the case of somatostatin,
physectomy and aging affected
nic LH-RH as indicated by anal-
ance.

absence and excess of T_4 on the
ıic somatostatin, LH-RH, serum
TSH levels in rats. As shown in
ere was no significant difference
othalamic somatostatin and the
ıntents between the control rats
5.2 ng, respectively), the thyroid-
rats (63.9 and 4.6 ng, respec-
thyroidectomized rats treated
g of T_4 (65 and 3.3 ng, respec-
the rats treated with 10 μg of
ınd 4.2 ng, respectively). The
n TSH concentration in the thy-
ed rats (4.5 ± 0.4 μg/ml) and in those thyroidectomized and treated with 0.1 μg of T_4 (3 + 0.2 μg/ml) were significantly higher than the serum TSH level in the control rats (0.57 ± 0.04 μg/ml), whereas the serum TSH levels in the normal rats treated with 10 μg of T_4 (0.4 ± 0.05 μg/ml) were significantly lower than the control ($P < 0.05$). The GH levels were not significantly different between the groups, due to considerable variation in each group.

Effect of urethane and Nembutal anesthesia on hypothalamic somatostatin and LH-RH content. Five min after urethane injection, the hypothalamic content of somatostatin appeared to rise slightly, but this was not statistically significant. The somatostatin decreased significantly ($P < 0.05$) 10 min after injection and then showed a rebound. The serum GH levels fell rapidly immediately after injection of urethane and remained at a low level after 10 min.

On the other hand, the hypothalamic somatostatin content decreased significantly ($P < 0.05$) 5 min after injection of Nembutal, which was associated with an apparent rise of serum GH. It then rose gradually for 60 min after injection and then decreased. Although, due to a large variation of serum GH in each group, the difference in GH between the groups did not reach a significant level, the pattern of change in the hypothalamic somatostatin was close to a mirror image of the pattern of change in

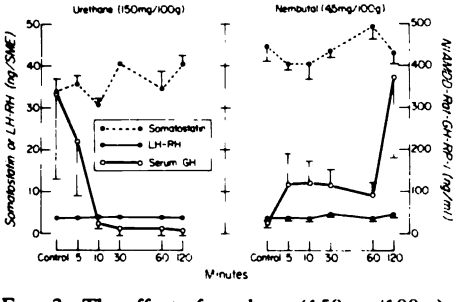


FIG. 2. The effect of urethane (150 mg/100 g) and Nembutal (4.5 mg/100 g) on hypothalamic LH-RH contents, somatostatin contents, and serum GH levels. The concentration of hypothalamic somatostatin 10, 30, and 120 min after urethane injection were significantly different from those of the control level ($P < 0.05$). The hypothalamic somatostatin contents 5, 10, and 60 min after Nembutal administration were significantly different from the control level ($P < 0.05$).

TABLE I. EFFECT OF ABSENCE OR EXCESS OF THYROID HORMONE ON HYPOTHALAMIC LH-RH AND SOMATOSTATIN CONTENTS AND SERUM PITUITARY HORMONE LEVELS

	Hypothalamic LH-RH (ng/SME)	Hypothalamic somatostatin (ng/SME)	Serum GH (ng/ml)	Serum TSH (μg/ml)
Control male rats	5.2 ± 0.4(8)[a]	62.5 ± 3(8)	21.0 ± 10(9)	0.57 ± 0.04(7)
Thyroidectomized rats (T̄)	4.6 ± 0.3(8)	63.9 ± 4(7)	14.8 ± 10(7)	4.5 ± 0.4 (6)
T̄ + 0.1 μg T_4	3.3 ± 0.5(6)	65.0 ± 4(6)	8.6 ± 5(5)	3.0 ± 0.2 (5)
Normal rats + 10 μg T_4	4.2 ± 0.3(9)	65.7 ± 4.3(10)	15.1 ± 14(9)	0.4 ± 0.06 (9)

[a] Mean ± SE. The number in parenthesis represents the number of animals in each group.

the serum GH levels. The hypothalamic content of LH-RH did not change after injection of these anesthetics.

Discussion. The present study indicates that hypophysectomy results in a decrease in both somatostatin and LH-RH contents of the hypothalamus in rats. In other words, the removal of the target organ, the pituitary gland, resulted in a decrease in both the inhibiting hormone for GH and TSH and the stimulating hormone for LH and FSH. The results are in agreement with findings by Baker and Yen (17) for somatostatin, and Baker and Dermodey (18) for LH-RH, as examined by immunocytochemical methods, and with the finding by Wakabayashi *et al.* (19) for somatostatin as examined by RIA. Bassiri and Utiger (20) reported that hypothalamic TRH also decreased after hypophysectomy. Reduction of the hypothalamic hormone content may be the result of an increased release and/or a decreased synthesis, or a greater rate of secretion than those of synthesis, regardless of their magnitudes. It is unknown which of these conditions resulted in posthypophysectomy decrease in hypothalamic hormones. Although Nallar and McCann (21) reported that bioassayable LH-RH in the plasma increased to a detectable level after hypophysectomy, we could not confirm their results (unpublished observation). Since hypophysectomy reduced the contents of both release inhibiting and release stimulating hormones in the hypothalamus, it is inconceivable that the reductions of these hypothalamic hormones resulted from a short feedback action provoked by the removal of pituitary hormones. Rather, these reductions appear to have been caused by decreased synthesis associated with extensive degeneration of the hypothalamic hypophysial nerve tract following surgery.

However, a significant difference between the changes in hypothalamic somatostatin and LH-RH was observed in long-term hypophysectomized rats. Somatostatin progressively decreased up to 2.5 months after hypophysectomy, whereas LH-RH did not decrease further after the 24th postoperative day. Hypothalamic TRH decreased to 54% of the control after 2 weeks but was restored to 74% of the control after 4 weeks, which could be the result of regeneration of the neural tissue (20). Baker and Yen (17) also reported that the greatest depletion of somatostatin in rats was observed in the medial caudal region of the hypothalamus, where the regeneration of the neural tissue had occurred. Therefore, the progressive decrease of somatostatin even after the neural tissue was generated in contrast to the changes in LH-RH and TRH should be more meaningful if any short feedback influence were to be considered. It is possible that the synthesis of somatostatin in the hypothalamus may require a certain level of GH and/or TSH in circulation, whereas synthesis of LH-RH and TRH may not need gonadotropins and TSH, respectively.

Although somatostatin appears to be involved in the physiological regulatory mechanism of TSH secretion (12, 22, 23), its level in the hypothalamus did not change by either excess or removal of the thyroid hormones. Thyroidectomy and excess T_4 did not modify the hypothalamic LH-RH content either, although such a condition is known to affect the pituitary gonadal function (4). Hypothalamic TRH does not change under similar conditions (20).

On the other hand, urethane and Nembutal modified the somatostatin content but

he LH-RH content in the hypothala-
The rapid change of hypothalamic
tostatin could reflect the rapid change
: turnover rate of this hormone. After
ane injection, the somatostatin re-
:d at the same level or appeared to
a slight rise at 5 min, and decreased
icantly at 10 min. The pattern resem-
that of hypothalamic corticotropin re-
g factor (CRF) after stress. The
;e of hypothalamic CRF after stress
nterpreted as the result of a rapid
ıse of synthesis associated with an en-
:d release since plasma ACTH rose
ly (24). Since the apparent rise or the
ained level of hypothalamic somato-
5 min after urethane injection was
iated with a rapid decrease of serum
this could also be interpreted as a
increase of synthesis associated with
hanced release of somatostatin.

the other hand, the hypothalamic
tostatin curve was almost the mirror
: of the serum GH curve during Nem-
anesthesia. However, in this experi-
the initial GH levels were low and
ɔse slightly following injection of Nem-
. It is not clear whether this relation-
s related to the change in the turnover
pothalamic somatostatin and GH se-
ın.

nmary. Twenty-four days after hy-
ysectomy both hypothalamic somato-
and LH-RH contents decreased to $^1/_3$
: control values in male rats. The LH-
:mained unchanged thereafter, whereas
ɔmatostatin continued to fall until 2.5
hs after hypophysectomy. The hypo-
nic somatostatin and LH-RH contents
7 day old rats (24.5 and 6.0 ng,
:tively) were significantly lower than
in 59-day-old rats (49 and 9.1 ng,
:tively).

yroidectomy and excess of T_4 did not
:y the hypothalamic somatostatin in
:H content. On the other hand, an
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ypothalamic somatostatin content, but
he LH-RH content. After urethane
ion the somatostatin content did not
;e at 5 min as compared with the level
ime, but showed a decrease at 10 min.
was associated with a rapid fall of serum GH level which remained low there-
after. The hypothalamic somatostatin then increased stepwise. Nembutal caused a de-
crease of the hypothalamic somatostatin content at 5 and 10 min and an increase at
60 min. The pattern of change in the hypo-
thalamic somatostatin levels resembled a mirror image of the patterns of change in
the serum GH levels.

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rial assistance.

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OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 241-244 (1978)

ion of Circulating Immunoreactive Human Parathyroid Hormone: Comparison with a 1-34 N-Terminal Synthetic Fragment (40029)

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sequence of the 1-34 amino terminal ically active fragment of human para- d hormone (hPTH) is not unequivo- lucidated as three residues are contro- . (1, 2). Immunochemical studies in- g these two sequences have yielded /hat conflicting results. Fisher *et al.* ısing antibodies raised against ex- d hPTH, showed a complete cross- ɔn between extracted hPTH and the N-terminal fragment synthesized ac- g to the structure proposed by Brewer (1-34 hPTH B) (1). Segre and Potts ;ing several different heterologous an- ɛs raised against bovine parathyroid ɔne, showed that 1-34 fragment syn- ɛd according to the sequence pro- by Niall *et al.* (2) (1-34 hPTH N) ghly purified hPTH displaced the bo- TH tracer in an identical manner. In ;ystem, low or no displacement was ɛd with 1-34 hPTH B.

have shown (5) that extracted hPTH munochemically identical to 1-34 N, using a homologous radioimmu- ıy specific for this peptide. This ra- nunoassay could eventually be used e measurement of hPTH present in ; or plasmas. It was however essential npare 1-34 hPTH N with circulating , preferably normal. Therefore we ɛxtracted hPTH from normal plasma inity chromatography and compared nunochemical properties with the syn- peptide. Simultaneous comparisons ılso carried out with extracted hPTH,)uso extracts of adenoma cultures and ɔlasma obtained from hyperparathy- atients.

erials and methods. Homologous RIA 34 hPTH N. A sensitive radioimmu- ıy (7 pg per tube) was developed using ɔdies raised in the goat against the 1- TH N. The synthetic peptide (5) was labeled with ^{125}I using the chloramine T method (6). The labeled tracer was purified by Quso G.32 (7) and by chromatography on Sephadex G-25 (Pharmacia). Fifty picograms of the tracer was incubated in barbital buffer, pH 8.6, 0.05 *M*, containing 17% (by volume) of normal human plasma to minimize adsorption of hormone, sodium azide (0.2%), and antibody G10/017 at a final dilution of 1/250,000 and increasing amounts of unlabeled peptide (0.008-16 ng). All preparations tested were lyophilized and dissolved in the standard buffer, and multiple dilutions were added in the radioimmunoassay. Final incubation volume was 500 μl and incubation was carried out for 7 days at 4°. Separation of bound and free tracer was achieved by dextran-coated charcoal (8).

Affinity chromatography. Affinity chromatography was performed using either antibodies against the 1-34 hPTH N (column 1) or antibodies against bovine PTH (column 2). The two types of antibodies were selected for their high cross reaction with hPTH. Coupling to Sepharose 4B (Pharmacia) was performed by the cyanogen bromide technique (9) according to the procedure we have used for anticalcitonin antibodies (10). At the end of coupling, unreacted material was removed by several alternate washes of standard buffer and acetic acid 1 *M*. The coupled antibodies were finally resuspended in the standard buffer and packed in two small chromatographic columns (Ø: 0.9 cm, L: 15 cm). Specificity of the affinity columns was established by the specific binding of labeled 1-34 hPTH N (column 1) or labeled bovine PTH (column 2), and by the absence of binding of unrelated labeled peptides such as porcine, salmon or human calcitonin. Nonspecific binding never exceeded 5%. The antibovine antibodies used in column 2 were predomi-

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nantly C-terminal as they did not retain the labeled 1-34 hPTH N.

Plasma extracts. Aliquots (300 ml) of normal plasma were passed on each column; after a distilled-water wash, fixed immunoreactive material was eluted using 1 *M* acetic acid. In order to minimize the adsorption of immunoreactive material, effluents of the columns were directly collected on vessels cooled by dry-ice. The acid extracts obtained from both columns were separately freeze-dried and dissolved in 10 ml of distilled water; the purification factor was 300. Affinity chromatography (column 1) was also performed on plasma samples obtained from a totally parathyroidectomized patient (PTX) and a hyperparathyroid subject.

Tissue extracts. Human parathyroid adenomas obtained by surgery and immediately frozen in dry ice were pooled, defatted, and extracted by the phenol-TCA method (11). Quso extracts of adenoma culture medium were obtained from the British Medical Research Council. In addition, extracts of human kidney and muscle were prepared. Protein contents of the extracts were determined by the Folin-Wu technique.

Statistical treatment of data. The ratio of bound tracer to bound tracer at O dose of cold hormone (B/Bo) was computed for each dose level. Radioimmunoassay curves were linearised using the arc Sine transform of $\sqrt{B/Bo}$ and the log of the dose. Regression curves were then performed using the method of least squares.

Results. Figure 1 shows that whether immunoreactive material present in normal plasma is extracted by antibodies directed towards the 1-34 hPTH N column 1 (N terminal) or bPTH (column 2) multiple dilutions of the two extracts are superimposable on the standard curve. The only difference is that total amount of immunoreactive material extracted by column 1 (anti 1-34 hPTH N) was four times higher for the same sample of normal plasma than the material extracted by column 2 (anti bPTH).

Concentration by affinity column of nonspecific factors interfering with the assay could be excluded as the extract of 30 ml of

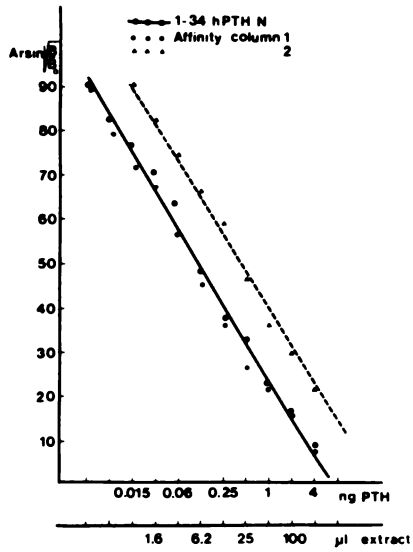

FIG. 1. Cross reaction of hPTH extracted from normal plasma by affinity column anti 1-34 hPTH N (★) or anti bPTH (▲), and 1-34 hPTH N (●) in a homologous radioimmunoassay: antiserum goat 10/017 1/250,000 anti 1-34 hPTH N, ^{125}I labelled I-34 hPTH as tracer.

PTX plasma was unable to displace the tracer; in contrast the extract of a hyperparathyroid plasma (30 ml) shows total cross-reaction (Fig. 2). In addition, multiple dilutions of a plasma obtained from a hyperparathyroid patient (Fig. 2) as well as extracts of adenomas or Quso extract of adenoma culture medium (Fig. 3) were superimposable on the standard curve. On the other hand, no displacement of the tracer was observed with extracts of muscle or kidney, even at concentration 10^4-fold higher than the adenoma extract.

Discussion. The total cross-reaction observed with hPTH extracted from adenoma or tissue culture medium of human adenoma and the 1-34 hPTH N using a homologous assay for this fragment suggests that the natural hormone or its N-terminal fragments share common antigenic sites with the peptide synthesized according to the sequence proposed by Niall *et al.* (2). Such common antigenic sites are also present in the hormone or its fragments circulating in hyperparathyroid plasma. In the case of normal plasma, concentration of immunoreactive material was a necessary prerequisite before carrying out cross reaction stud-

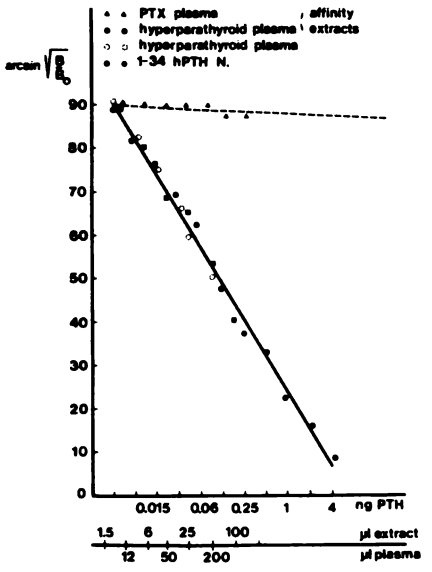

FIG. 2. Displacement of labelled 1-34 hPTH N by hyperparathyroid plasma (○), extracts from plasma of hyperparathyroid patient (■) or from plasma of a parathyroidectomized subject (▲) and 1-34 hPTH N (●) in a homologous R.I.A. anti 1-34 hPTH N.

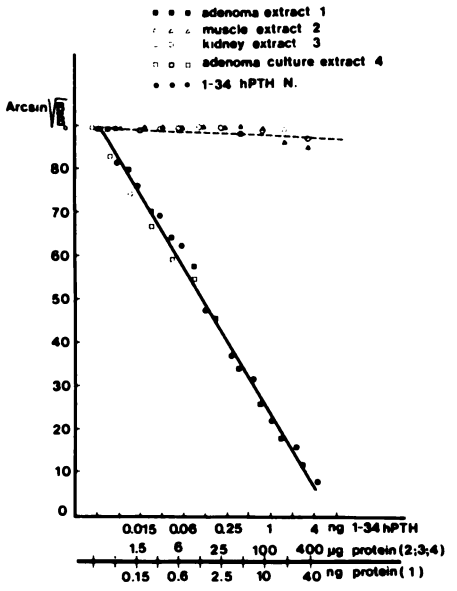

FIG. 3. Cross reaction of extracts from adenoma (■), adenoma culture medium (□), muscle (△) or kidney (○) and 1-34 hPTH N (●) in a homologous R.I.A: anti 1-34 hPTH N.

ies due to the low amount of immunoreactive hormone present. Extraction by column 1 (anti 1-34 hPTH N) shows that in the case of normal plasma, hPTH or some of its fragments are immunochemically identical to fragment 1-34 hPTH N. Immunoreactive material present in this extract could comprise 1-34 sequence of either smaller or larger peptides involving the N terminal antigenic site. Column 2 does not retain molecules $\leq$ 1-34 sequence and thus the antibodies coupled are presumably directed towards mid or C terminal regions of the molecule or its circulating fragments, and the extract tested was therefore devoid of molecules $\leq$ 1-34. The result of the radioimmunoassay of this extract and the total cross reaction observed between this extract and the 1-34 hPTH N demonstrates the presence in normal plasma of peptides > than 1-34 and intact molecules. This implies that radioimmunoassay using antibody G10/17 will measure all the biologically active fragments present in peripheral plasma and probably certain biologically inactive molecules in which N terminal amino acids may be missing. As we have already pointed out, extraction by affinity chromatography is not total as fragments of hPTH or even the intact hormone in which the antigenic sites are either masked or lacking will escape fixation. Notwithstanding these limitations, the affinity chromatography is usefull for the concentration and purification of immunoreactive species present in blood as well as for obtaining plasma free from immunoreactive material. We now use such plasma for minimising adsorption of the hormone instead of normal plasma in the standard assay.

Our results further substantiate the studies of Segre and Potts (4) which showed with heterologous radioimmunoassays, identical immunochemical reaction of 1-34 hPTH N and 1-84 hPTH. In addition our results extend this identity to hPTH or its fragments present in normal or hyperparathyroid plasma. Our findings do not at present disagree with those obtained by Fisher *et al.* (3) i.e., the immunochemical similarity of 1-34 hPTH B and 1-84 hPTH, as the antiserum used shows a high cross reaction with 1-34 hPTH B synthesized in our laboratory (12). Furthermore Fisher *et al.* (3) did not study the cross reaction of 1-34 hPTH N in their radioimmunoassay sys-

tems, and thus their antibodies like ours could have been directed toward common sequences of the two proposed structures.

The results obtained in this study imply that hPTH or its N terminal fragments present in parathyroid gland or in blood share in their N terminal region a high degree of similarity in their tertiary structure with the sequence proposed by Niall *et al.* (2). Though the exact structure of the 1-34 hPTH is still debated, the results reported here indicate that certain antibodies raised against the 1-34 hPTH N can be used in establishing radioimmunoassays specific for circulating hPTH or its fragments. Preliminary results using this radioimmunoassay show that immunoreactive hPTH can be measured in normal plasma in 65% of cases, and that a good discrimination between normal and hyperparathyroid plasmas is observed (13).

Summary. Immunoreactive human parathyroid hormone (hPTH) was isolated from normal plasma by Sepharose bound antibodies raised against either the synthetic 1-34 fragment of hPTH (Niall sequence) or bovine PTH. The isolated material was compared with extracted hPTH, Quso extracts of adenoma cultures, hyperparathyroid plasma and the synthetic fragment using a radioimmunoassay homologous for this fragment. Complete cross reaction of all preparations studied was observed in this system, indicating that immunoreactive material separated from normal plasma by this method was identical with the synthetic peptide and implying that hPTH or its fragments present in normal and hyperparathyroid plasma, in tissue extracts or released in culture medium share common tertiary structures with the 1-34 peptide, synthetized according to the structure proposed by Niall *et al.*

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cts of the Oral Converting Enzyme Inhibitor (SQ 14225) on One-Kidney Hypertension in the Dog (40030)

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recent preliminary studies (1, 2), nonstrated that chronic one-kidney ion developed in the dog despite is angiotensin blockade during the h renin phase of this experimental The angiotensin II antagonist, i[8]] angiotensin II, and the convert- ie inhibitor, SQ 20881, were given isly for 1 and 2 days before and 7 days after renal artery constric- ing [Sar[1], Ala[8]] angiotensin II in-), arterial pressure appeared to during days 2-4 after renal artery on but the change was not signifi- fferent from the level of arterial during analog infusion in normal ogs until the fifth day after renal enosis. At this time, the level of ressure achieved was characteristic ironic hypertensive state. During 1 infusion (2), arterial pressure ignificantly elevated until the sixth renal artery constriction. This de- development of hypertension dur- 0881 infusion appeared to result iotensin blockade; also, a possible it rise in the plasma level of bra- ind the associated depressor influ- this peptide was considered. As he SQ 20881 infusion was discon- rterial pressure increased further chronic hypertensive state was . The findings demonstrate that the giotensin system need never be ac- or the development and mainte- chronic renovascular hypertension. present study, the new oral con- nzyme inhibitor, SQ 14225, was dogs 1 day before and 7 days after ery stenosis to evaluate further the f converting enzyme inhibition. me converts angiotensin I to angi- I and degrades bradykinin. SQ ven orally to rats inhibited the pressor effect of angiotensin I, augmented the depressor action of bradykinin and lowered arterial pressure in experimental renin-dependent renovascular hypertension (3); also McCaa (4) has a preliminary report to show that SQ 14225 lowered arterial pressure and plasma aldosterone concentration and increased plasma renin activity (PR) in conscious sodium-deficient dogs. The purpose of the present study was to determine the efficacy of orally administered SQ 14225 on the development of experimental one-kidney hypertension in dogs and to compare this response to that obtained earlier (1, 2) with [Sar[1], Ala[8]] angiotensin II and SQ 20881.

Methods. Three female hounds (15-19 kg) were unilaterally nephrectomized under sterile conditions and catheters were placed in a femoral artery and vein for chronic studies; the catheters were exteriorized between the shoulder blades. The animals were allowed to recover for several days and they were trained to lie quietly on a table. Daily measurements of arterial pressure and heart rate were made each morning between 8:00-8:30 with a Statham pressure transducer and a Sanborn recording system, and blood was drawn for determination of plasma electrolytes, PRA and plasma aldosterone concentration. The blood samples were collected in tubes containing 0.1 ml of 10% ethylene-diaminetetraacetic acid (EDTA) per 10 ml of blood; the samples were cooled in an ice bath and centrifuged in cold. Plasma was stored frozen until time of radioimmunoassay for PRA and aldosterone. Plasma was prepared for generation of angiotensin I by dialyzing it against a phosphate buffer (pH 5.4) for 18 hr (three changes); diisopropylflourophosphate (DFP) and sodium chloride were added to the plasma prior to incubation at 37° for 60 min. The reaction was then stopped by

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placing the tubes in a bath of ice-water. Angiotensin I content was quantified by radioimmunoassay (5) and PRA is expressed as nanograms of angiotensin I/ml/hr. Plasma aldosterone concentration was determined by the method of Bühler, Sealey and Laragh (6); aldosterone was separated by celite column chromatography and quantified by radioimmunoassay. The dogs were fed a daily diet containing 60–65 mEq sodium and were housed in metabolic cages for the determination of sodium balance.

After measurements were made for two days, the converting enzyme inhibitor SQ 14225 (D-2-methyl-3-mercaptopropanoyl-L-proline) was given orally for 8 days in divided doses at 8:30 AM, 4:30, and 10:30 PM. The efficacy of SQ 14225 in producing blockade of angiotensin I was assessed daily before the morning dose by determining the pressor response to 2 and 4 μg of intra-arterial injections of exogenous angiotensin I; the peptide was given intra-arterially rather than intravenously because an indwelling arterial catheter was available. On the second day of SQ 14225 administration, the renal artery was constricted to reduce renal blood flow by 55–60%, a stenosis sufficient to produce benign one-kidney renal hypertension in dogs within 24 hr. (7). Administration of SQ 14225 was continued for 7 days after renal artery constriction to encompass the entire early high renin phase of renal hypertension and measurements were continued. Observations were made during a 5-day recovery period.

The first two dogs studied, received 10 mg/kg of SQ 14225 three times daily for the day before and for 5 days after renal artery constriction. Occasionally, a slight pressor response to the 4 μg dose of angiotensin I was observed. Consequently, the 10:30 PM dose of SQ 14225 was increased from 10 to 15 mg/kg for the remaining two days of angiotensin blockade. In the third dog, 15 mg/kg of SQ 14225 was given at 10:30 PM throughout the study so the total dose was 35 mg/kg/day.

Dose response curves for the depressor action of bradykinin triacetate were obtained by giving bolus intravenous injections from 0.1 to 30 μg/kg to all three dogs before and to two of the three animals on the sixth day of SQ 14225 administra

Data from all experiments were anal by use of the Student's paired t test, cr to a 5% level of significance.

Results. The completeness of angiot blockade was demonstrated by finding slight pressor responses to injections o giotensin I during SQ 14225 administra Before the inhibitor was given, 2 μ angiotensin I increased arterial pressu 23 ± 2.0 mm Hg while 4 μg gave a resp of 32 ± 2.8 mm Hg.; during SQ 1 administration, a response of 6.5 ± mm Hg occurred in 13% of the 2 μg i tions and a response of 7 ± 1 mn occurred in 50% of the 4 μg injections

During the first day of angiotensin b ade and before renal artery constri mean arterial pressure decreased 9 mr ($P < 0.05$) but the changes in PRA plasma aldosterone concentration wer significant (Table I). During continue giotensin blockade and after renal a stenosis, arterial pressure continued t significantly decreased on day 1 bu creased slightly each day thereafter the fifth day at which time arterial pre was significantly elevated above the cc level. Arterial pressure was sustained a hypertensive level for the remaining 2 of SQ 14225 administration and durin recovery period. PRA was increased s icantly throughout the period of coml angiotensin blockade and renal artery nosis and during the first recovery Plasma aldosterone concentration rem at the normal control level throughou study. Sodium excretion appeared tc crease on the day following renal a constriction but the variability in resp among the three dogs was enough to vent a significant change from occurrin

To assess the possibility of a coincid increase in the plasma bradykinin level ing SQ 14225, dose response curv bolus injections of bradykinin triac were obtained before and during dru ministration (Table II). A striking inc in both the magnitude and the length c depressor responses occurred durin 14225 administration.

Discussion. During the last few substantial evidence has accumulate

TABLE I. EFFECTS OF ORAL ADMINISTRATION OF SQ 14225 BEFORE AND DURING THE ACUTE PHASE OF RENOVASCULAR HYPERTENSION IN THE DOG

	Days	Mean arterial pressure (mmHg)	Plasma renin activity (ng-Angl/ml/hr)	Plasma aldosterone level (ng%)
	Control 1	110 ± 5		
	Control 2	106 ± 2	0.7 ± 0.2	
	Control 3	103 ± 4	1.7 ± 0.9	5.3 ± 0.3
↑	1	97 ± 5*	6.4 ± 2.3	3.8 ± 0.3
	RAC			
SQ 14225 (30–35 mg/kg/day)	1	101 ± 1*	10.4 ± 3.2*	3.7 ± 0.7
	2	103 ± 2	10.9 ± 1.1*	4.5 ± 0.3
	3	115 ± 8	7.7 ± 1.1*	5.8 ± 0.7
	4	115 ± 7	6.5 ± 1.6*	4.5 ± 1.0
	5	118 ± 5*	4.3 ± 1.0*	3.5 ± 0.4
	6	116 ± 3*	5.0 ± 0.9*	3.6 ± 0.5
↓	7	121 ± 3*	4.9 ± 0.4*	4.5 ± 0.3
	Recovery			
	1	121 ± 4*	3.6 ± 0.1*	5.8 ± 0
	2	124 ± 3*	3.3 ± 0.1	3.5 ± 0.4
	3	122 ± 1*	1.7 ± 0.1	6.0 ± 1.2
	4	124 ± 2*	1.8 ± 0.1	3.8 ± 0.4
	5	123 ± 6*	1.8 ± 0.6	5.6 ± 0.4

* Denotes that value is significantly different ($P < 0.05$) from the average of the last 2 control values. The values for all three functions are presented as means ± SEM.

TABLE II. EFFECTS OF INTRAVENOUS BRADYKININ ADMINISTRATION BEFORE AND DURING ADMINISTRATION OF 35 MG/KG/DAY OF SQ 14225 IN THREE DOGS

		Bradykinin triacetate (μg/kg) as a bolus injection				
		0.1	1.0	3.0	10.0	30.0
I. Prior to SQ 14225 administration						
dog 1	Δ MAP[a]	8	15	23	29	40
	Duration[b]	21	15	21	81	102
dog 2	Δ MAP	9	25	30	38	40
	Duration	6	12	36	42	204
dog 3	Δ MAP	9	19	27	35	–
	Duration	6	18	24	180	–
II. During oral SQ 14225 administration (35 mg/day)						
dog 1	Δ MAP	27	32	49	–	–
	Duration	24	108	300	–	–
dog 2	Δ MAP	29	54	–	–	–
	Duration	21	300	–	–	–

[a] Mean arterial pressure (mmHg).
[b] Duration of depressor response (sec).

show increased activity of the renin-angiotensin system during the acute phase of both one and two-kidney hypertension in several experimental animals (8). PRA is increased for 3–6 days after renal artery constriction in the unilaterally nephrectomized dog and during this time the animals show a depressor response during bolus injections or brief periods of infusion of angiotensin II antagonists and converting enzyme inhibitors (8). This fall in arterial pressure from the hypertensive level toward or to normal indicates a pathogenic role for the renin–angiotensin system. On the other hand, angiotensin blockade throughout the entire 3- to 6-day acute high renin phase of hypertension failed to prevent the development of chronic one-kidney renovascular hypertension in the dog (1, 2).

The present observations confirm the

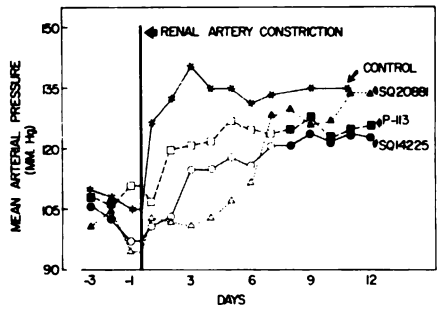

FIG. 1. Effects of angiotensin blockade on the development of one-kidney hypertension in dogs. The open symbols indicate the time of drug administration. The control group of 6 dogs was treated identical to the present experimental group receiving SQ 14225 except that no drug was given (7). The results with SQ 14225 show the present data on a dose of 35 mg/kg/day given orally to three dogs. The angiotensin antagonist, [Sar^1, Ala^8] angiotensin II (P-113) (1) and the converting enzyme inhibitor, SQ 20881, (2) were given intravenously by constant infusion at doses of 1 μg/kg/min (six dogs) and 10 μg/kg/min (five dogs) respectively.

findings reported earlier (1, 2) that the renin-angiotensin system need never be activated for the development and maintenance of chronic renal hypertension. Indeed, the blood pressure response to renal artery constriction during SQ 14225 administration was similar to that reported (1) with [Sar^1, Ala^8] angiotensin II (Fig. 1) except that the pressor response to renal artery constriction during SQ 14225 was slightly slower and somewhat less than with [Sar^1, Ala^8] angiotensin II; with both drugs, however, arterial pressure was not significantly elevated until the 5th day after renal artery stenosis. At this time and, thereafter, a chronic hypertensive state was present. The arterial pressure response during SQ 20881 infusion and after renal artery constriction (2) was less than that observed with [Sar^1, Ala^8] angiotensin II and with SQ 14225; not until the last day of SQ 20881 infusion was arterial pressure increased but an abrupt further increase in pressure occurred on the day following discontinuation of the drug and this was sustained. This slower and smaller response to renal artery stenosis during SQ 20881 and SQ 14225 compared with [Sar^1, Ala^8] angiotensin II might possibly be related to an increase in the plasma bradykinin level and the associated depressor activity with the SQ compounds. A significant fall in arterial pressure of 9 mm Hg occurred after 24 hr of SQ 14225 administration before the renal artery was constricted. Also, dose response curves to bolus injections of bradykinin revealed a much greater and more prolonged fall in blood pressure during SQ 14225 administration than occurred during the control observations. The smaller response to renal artery stenosis during SQ 20881 compared with SQ 14225 probably reflects more complete blockade of converting enzyme during the continuous infusion of SQ 20881; in the present dogs receiving SQ 14225, arterial pressure was measured before the 8:30 AM dose and this was 10 hr after the previous 10:30 PM dose of the drug. Consequently, a higher drug level might have been present during the SQ 20881 than SQ 14225 at the time of the arterial pressure measurements and resulted in a lower level of arterial pressure.

The responses in PRA were very similar with SQ 20881 and SQ 14225. No increase in plasma aldosterone concentration occurred during SQ 14225 administration and renal artery stenosis, a finding which demonstrates a marked degree of angiotensin blockade. It should be emphasized that SQ 14225 is a competitive antagonist of the converting enzyme so that blockade was never fully complete. Also, from data on the reduction or abolition of the pressor responses to bolus injections of angiotensin I, it is not possible to state the completeness of angiotensin blockade. For example, the degree of blockade necessary to block completely a pressor response to 2 μg of angiotensin I might be more than adequate to achieve 95-98% blockade of the action of the endogenous angiotensin II.

Thus, the use of SQ 14225 to achieve angiotensin blockade provides strong support for the concept that the renin-angiotensin system need never be activated for chronic one-kidney renovascular hypertension to develop and to be sustained. Furthermore, the present study minimizes the importance of the renin-angiotensin system in the pathogenesis of one-kidney hypertension. These findings point out the need to

search for another renal pressor mechanism or a neurogenic mechanism during the development of renovascular hypertension. A promising approach is the recognition of a new hypertensive substance called renopressin which has been described by Skeggs and associates (9).

Summary. Angiotensin blockade was produced one day before and for 7 days after renal artery stenosis in three unilaterally nephrectomized dogs by administration of SQ 14225. The completeness of angiotensin blockade was demonstrated by reduced or absent pressor responses to exogenous angiotensin I and by a normal plasma aldosterone level after renal artery stenosis. A depressor response (9 mm Hg) during the day before renal artery constriction and markedly exaggerated depressor responses to exogenous bolus injections of bradykinin after several days of SQ 14225 administration might reflect an increased plasma level of bradykinin but additional studies are needed on this idea. The arterial pressure response to renal artery constriction was markedly diminished during SQ 14225 and arterial pressure was not significantly elevated until the fifth day. PRA was elevated throughout the period of SQ 14225 administration and renal artery constriction. These findings confirm the earlier observations that the renin-angiotensin system need never be activated for the development and maintenance of chronic one-kidney renovascular hypertension.

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Interaction Between Human Hemoglobin Variants and Hemoglobin S (40031)

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Data from optical and ultrastructural studies have offered considerable insight into the location of contacts between hemoglobin molecules, and additional information concerning these intermolecular bonds has come from studies of factors which promote or inhibit gelation of Hb S. Of particular interest are the results of studies of the effects of specific amino acid substitutions at the intermolecular surface.

Several hemoglobin variants, such as the β chain variants Deer-Lodge (2 His → Arg), C (6 Glu → Lys), C-Harlem (6 Glu → Val, 73 Asp → Asn), G-Makassar (6 Glu → Ala), Leiden (6 or 7 Glu → O), J-Baltimore (16 Gly → Asp), N-Baltimore (95 Lys → Glu), O-Arab (121 Glu → Lys), D-Los Angeles (121 Glu → Gln), and the α chain variants I-Philadelphia (16 Lys → Glu) and Memphis (23 Glu → Gln) have been studied with regard to their influence on the gelation and solubility of deoxy Hb S (1-9). The data have shown that variants influencing the gelation or the solubility of deoxy Hb S or both have a substitution of one of the following residues: Asp 73β, Glu 121β, Lys 16α, and Glu 23α (4, 10-12). The substitution of a specific amino acid residue can weaken the interaction between the molecules (e.g. the Asp → Asn substitution at 73β) or strengthen the intermolecular contact (e.g. the Glu → Lys substitution at position 121β).

Recently we were able to study the effect of additional variants together with some variants already investigated, both in the deoxy- and oxyconformation; these include the three substitutions at position 121β, namely O-Arab (121 Glu → Lys), D-Los Angeles (121 Glu → Gln), Beograd (121 Glu → Val), and also E (β26 Glu → Lys), N-Baltimore (β95 Lys → Glu), I-Philadelphia (α16 Lys → Glu) and G-Georgia (α95 Pro → Leu). Hb C (β6 Glu → Lys) and Hb A were used as control.

Materials and Methods. Hemolysates were made from saline washed red cells. The hemoglobins O-Arab and C were obtained from patients homozygous for this variant, Hb Beograd from the patient with Hb Beograd β^0-thalassemia (13) (with about 90% of the variant), and Hb N-Baltimore from a patient with a Hb N-β^+-thalassemia (with 80% Hb N); red cell hemolysates were used without further treatment. The hemoglobins D-Los Angeles, E, G-Georgia and I-Philadelphia were isolated from red cell hemolysates of heterozygotes by DEAE-cellulose or by DEAE-Sephadex chromatography (14, 15). Red cell hemolysates from patients with sickle cell anemia were used as a source of Hb S; the level of Hb F (F_{AD}) in these samples was 4% or less.

Cyanferri hemoglobins were prepared with threefold excess of $K_3Fe(CN)_6$ and fourfold excess of KCN in 0.1 *M* phosphate buffer of pH 6.8; Sephadex G-25 chromatography was used to remove excess ferricyanide and KCN. The concentration of Hb was calculated from the millimolar extinction coefficient and absorbance at 540 nm after the sample was converted into cyanferrihemoglobin (16). Solutions of hemoglobin (either oxy- or cyanferri-) were concentrated by vacuum dialysis against distilled water using specific dialysis membranes (collodion bags #100 for protein concentration, capacity 8 ml, MW 25000, Schleicher and Schull, Inc., Lot #221/423); their visible spectra were verified using a Perkin Elmer double beam spectrophotometer (Coleman model 124 D). Minimum gelling concentration determinations were carried out at 25° on mixtures of deoxy Hb S and either the deoxy- or the cyanferri-derivative of other variants using the method of Singer and Singer (1) as modified by Bookchin *et al.* (5).

Results. The data, presented in Fig. 1,

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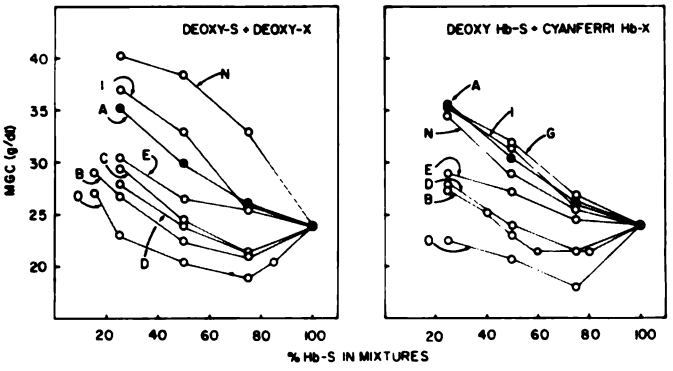

ɜ. 1. The effect of increasing amounts of various β chain and α chain variants on the minimum gelling ntration (MGC) of deoxy Hb S. Left panel: oxy derivatives of the hemoglobins N-Baltimore, I-Philadelphia, , C, D-Los Angeles, B (-Hb Beograd) and O-Arab mixed with oxy Hb S prior to deoxygenation; right : the cyanferri-derivatives of the same derivatives and of G-Georgia mixed with oxy Hb S prior to ygenation. Temperature 25°. The standard deviation for a mean gelling concentration of 24.40 was 1.38 an error of the mean 0.61. (n = 5).

irm and extend the data obtained by rs (1-3). The strongest positive effect observed for Hb O-Arab, followed by Beograd, Hb D-Los Angeles, and Hb he effect of Hb E was much less proıced and Hb I-Philadelphia and Hb Grgia affected the gelation of deoxy Hb a similar extent as Hb A. The effect of ı of the variants was the same whether ıe deoxy or oxy conformation, with the ption of Hb N-Baltimore. In the deoxy :, this variant exhibited a strongly negaeffect on the gelation of deoxy Hb S behaved like Hb A in the oxy (cyanferıtate.

iscussion. Five of the eight variants ied have a Lys → Glu (N, I) or a Glu ys (C, E, O-Arab) substitution. Substiın of Lys at 95β by Glu greatly deses intermolecular interaction, but a lar substitution at 16α has no effect. In rast, a Glu → Lys substitution appears ıcrease intermolecular interaction but effect is again greatly dependent upon location of the substitution. It has been med that Glu 121β forms a hydrogen ł with Lys 17β of the same chain; the r residue forms an intermolecular hyen bond with carbonyl oxygen of His β_2 of the neighboring molecule in the ɜ strand (17). Thus, substitution of Glu 3 by either Val or Gln or Lys would ken the bond between this residue in tion 121 and Lys 17β, and would make Lys 17β more accessible for the formation of intermolecular hydrogen bonds (17). The difference in effect between Hb O-Arab, Hb Beograd or Hb D-Los Angeles on the gelation of deoxy Hb S may be explained by the formation of an additional intermolecular hydrogen bond when a lysyl residue is introduced in position 121β. Similarly, substitution of a lysyl residue in position 95β by a glutamyl residue may result in the loss of a bond that participates in intermolecular interaction of deoxy hemoglobin molecule thereby decreasing the gelling interaction. Such an effect is not apparent for lysyl residue in position 16α.

These results support X-ray data (17) and extend the correlation observed (2) between the clinical severity of a number of sickling variants and the effect of the variants on the gelation of Hb S in mixtures.

Summary. Seven abnormal hemoglobins were investigated for an effect on the gelation of Hb S in the deoxy- or oxy-conformation. A positive effect (decreased MGC) was associated with substitution of Glu β6, Glu β26 and Glu β121. No difference was observed between the deoxy- or oxy-hemoglobins except in the case of Hb N-Baltimore which, in the deoxy state, exhibits a strongly negative effect on gelation but behaves like Hb A in the oxy state.

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ıolera Toxin Inhibits Antiviral and Growth Inhibitory Activities of Human Interferon (40032)

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ıt studies indicate that binding of ɔn to ganglioside or ganglioside-like haride surface receptors is a neces- t step for the establishment of anti- tivity (1-3). Cholera toxin and the ɔtein hormones thyreotropin (TSH) nan chorionic gonadotropin (hCG) gangliosides with similar but slightly t carbohydrate specificity. The mon- nglioside G_{M1} seems to be one spe- eptor for cholera toxin. The ganglio- ds cholera toxin and blocks its action h cholera toxin and the glycoprotein es inhibit the induction of antiviral interferon, supporting the notion of ı binding sites on the cell membrane

es its classical antiviral effect inter- are known to influence numerous functions, fe. cell growth, phago- tivity, antigen expression, etc. com- lescribed as nonantiviral activities. growth inhibitory activity is proba- most important and certainly the oroughly studied of these activities. ; reasonable to believe that reaction face membrane receptors might be ıon step in development of the var- ects of interferons. We, therefore, ɛd whether cholera toxin could in- ɛ growth inhibitory activity of inter- ıd correlated it to the inhibition of l activity. The present communica- ɔws that the two different activities ibited by the addition of cholera a comparable extent. Since most of ier studies were performed on mu- lls with murine interferon we ex- the phenomenon to include human l human interferon.

ials and methods. Cells. The human U-cell line was originally obtained r K. Cantell, Helsinki. The U cells own in Eagle's Minimal essential ı (MEM) with addition of 10% calf serum, and maintained in MEM with 5 or 2% calf serum.

Reagents. Cholera enterotoxin was purchased from Schwarz-Mann. A 10^{-5} *M* stock solution was prepared in Tris buffer and kept at −20°. Further dilutions were prepared in MEM immediately before use.

Interferon. Partially purified human leukocyte interferon was a gift from Dr K. Cantell, Helsinki. It was assayed by an infectivity inhibition microtest (6) employing U cells and Vesicular stomatitis virus (VSV). The titers are expressed in international standard reference units (69/19).

Antiviral activity was also assayed by the effect on virus yield. One day old U cells, about 10^5 cells per tube were treated with interferon and with cholera toxin as described in the results section. After incubation overnight the cells were washed and infected with 10^3 TCID of VSV. After 18 hr incubation the cultures were frozen and thawed and assayed for infectious virus yields in U cells. Cytopathic effect (CPE) was read after 72 hr incubation. The titers are given as the highest dilution showing CPE in half of the tubes determined by the method of Reed and Muench.

Assay of cell growth inhibitory activity. Culture tubes were seeded with 10^5 U cells in 1 ml of MEM with 2% calf serum, containing cholera toxin and interferon as indicated in the results section. The cultures were incubated at 37° in 5% CO_2 atmosphere. After 72 hr the monolayers were treated with trypsin–versene and the cells were counted in a hemocytometer. Each value given in the results represents the mean of at least five parallel cultures.

Results. The data in Fig. 1 show that interferon dose-dependently reduced the yield of VSV produced in U cells. When 10^{-9} or 10^{-7} *M* cholera toxin was added to the cells together with interferon the antiviral activity was reduced. With 50 units of

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interferon there was a significant reduction of interferon effect with both 10^{-9} and 10^{-7} *M* toxin. Addition of 10^{-7} *M* cholera toxin also reduced the antiviral titer of an interferon preparation tested in an infectivity inhibition microtest (Table I). This effect was more pronounced if the cells were pretreated with the toxin for 2 hr prior to addition of interferon. If toxin was added 2 hr after interferon no effect was observed.

The number of cultured U cells increased from 1×10^5 to $2.8\text{–}5.1 \times 10^5$ after 3 days incubation. Growth of U cells was dose-dependently inhibited by interferon (Fig. 2 and Table II). Addition of 10^{-9} *M* toxin greatly reduced this effect. Higher concentrations than 10^{-7} *M* of the toxin were slightly inhibitory itself, but 10^{-8} *M* alone had no effect on the cell growth. The effect of cholera toxin on the interferon-produced growth inhibition was more pronounced if the cells were pretreated prior to addition of interferon, and less if added after the interferon (Table II).

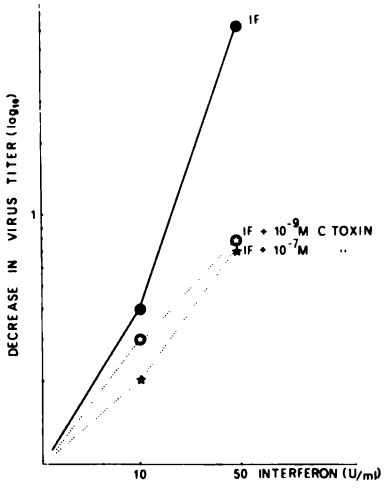

FIG. 1. Effect of cholera toxin on the antiviral activity of interferon. One day old U-cells, about 10^5 cells per tube, were treated with 10 or 50 units/ml of human leukocyte interferon with or without presence of 10^{-7} or 10^{-9} *M* cholera toxin or cholera toxin alone. The toxin was added together with interferon. After incubation overnight the cells were infected with 10^3 TCID of VSV. After 18 hr incubation the cultures were frozen and thawed and assayed for infectious virus yields.

TABLE I. EFFECT OF CHOLERA TOXIN ON INTERFERON TITER.[a]

10^{-7} *M* Cholera toxin	Interferon titer ($\log_{10}$ units)
0	2.85
Added together with interferon	2.45
Added 2 hr before interferon	1.65
Added 2 hr after interferon	2.85

[a] A sample of human leukocyte interferon was assayed by an infectivity inhibition microtest. Cholera toxin was added to the cells 2 hr before, at the same time or 2 hr after interferon and incubated overnight. The cells were then washed and infected with VSV. All cultures were examined for development of CPE after 3 days incubation.

Discussion. Cholera enterotoxin reduced two different activities of human interferon: induction of antiviral state and the cell growth inhibitory activity. The suggested mechanism of cholera toxin effect is an interaction with membrane surface receptors (1). Cholera toxin reacts with gangliosides, especially G_{m1} (4) and either directly or indirectly interferes with or blocks the interferon interaction with these structures (1). Alternative mechanism may be simply an alteration of the cell surface making the

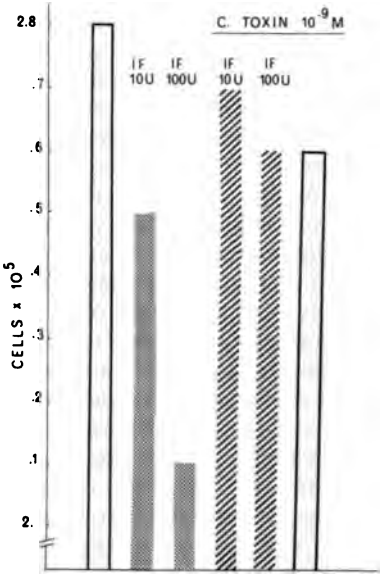

FIG. 2. Effect of cholera toxin on the cell growth inhibitory activity of interferon. U cells were seeded in Eagles MEM, about 10^5 cells per tube. Parallel tubes were added 10 or 50 units/ml of human leukocyte interferon, 10^{-9} *M* cholera toxin or interferon and toxin at the same time. After 3 days incubation the monolayers were trypsinized and the cells counted. The columns represent the mean of five tubes.

TABLE II. EFFECT OF INTERFERON AND CHOLERA TOXIN ON GROWTH OF U CELLS.[a]

Treatment of cells	Reduction of cell growth (% of control)
Interferon, 100 units	55.6 ± 8.6*
Interferon, 100 units + C. toxin, 10^{-9} *M*, same time	76.0 ± 6.8* [b]
Interferon, 100 units + C. toxin, 10^{-9} *M*, 2 hr later	59.0 ± 4.5** [c]
C. toxin, 10^{-9} *M* + Interferon, 100 units, 2 hr later	77.0 ± 3.6** [d] [b]
Interferon, 500 units	40.6 ± 4.0**
Interferon, 500 units + C. toxin, 10^{-8} *M*, same time	86.6 ± 7.6** [c]
C. toxin, 10^{-8} *M*	96
C. toxin, 10^{-9} *M*	98

[a] U cells were seeded in medium containing interferon, C. toxin or both. After 3 days incubation the monolayers were trypsinized and the cells counted.

[b] $P < 0.01$ compared with 100 units interferon, by *t* test.

[c] $P < 0.01$ compared with 100 units interferon + C. toxin same time.

[d] $P < 0.02$ compared with 100 units interferon + C. toxin 2 hr later.

[e] $P < 0.01$ compared with 500 units interferon.

* Mean of five experiments ± SD.

** Mean of three experiments ± SD.

membrane less receptible for interferon.

One has to consider the possible role of cyclic nucleotides. It has been suggested that cyclic AMP acts as a second messenger to substances which interact with the cell surface from without, fe. insulin and other hormones. Also interferon acts on the cell surface possibly without entering the cell (7-9), and it influences intracellular cAMP levels (10). Cholera toxin increases the accumulation of cAMP intracellularly (11). However, cAMP seems to have an enhancing effect on antiviral activity (12, 13) and usually an inhibiting effect on cell growth (14), the opposite of the observed effect of cholera toxin. It is therefore not very likely that the observed effect of cholera toxin is mediated by cAMP.

The effects on antiviral state and on cell growth were obtained by the same concentrations of the cholera toxin, and they had comparable kinetics suggesting that the step inhibited by cholera toxin is the same or related for the two different activities. Furthermore that cholera toxin blocks an early phase of the interferon action expressed both as antiviral activity and cell growth inhibitory activity. Finally the present findings, obtained with human cells and human interferon show that the effect of cholera toxin is not restricted to the murine system.

Summary. Cholera enterotoxin reduced the antiviral activity and the cell growth inhibitory activity of human leukocyte interferon. Both effects were more pronounced if the cells were treated with toxin before interferon and less if toxin was added after interferon.

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Effects of β-Adrenergic Amines on Frog Gastric Mucosa (40033)

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In a review article, Holton (1) states that both alpha and beta agonists in doses of the order of 1 mcg kg^{-1} min^{-1} inhibit acid secretion in conscious animals, that histamine-stimulated secretion is less easily depressed than other means such as pentagastrin stimulation, and that beta agonists in doses of the order of 0.1-0.5 mcg kg^{-1} min^{-1} increase histamine-stimulated secretion. However, much of the influence of adrenergic amines, *in vivo*, had been attributed to effects on mucosal blood flow (2). The isolated frog gastric mucosa enables the effects of catecholamines to be studied in the absence of a blood supply. Thorpe *et al.* (3) had, in fact, investigated the effects of catecholamines on the H^+ secretory rate of the isolated bullfrog gastric mucosa. They found that epinephrine and norepinephrine in concentrations as high as 5 m*M* produced statistically insignificant decreases in histamine-stimulated H^+ secretion. Recently Rose *et al.* (4) found that norepinephrine, when added to the nutrient solution of isolated gastric mucosae of Rana pipiens, produced a significant decrease of about 20% to about 100% respectively for concentrations increasing from 0.1 to about 2.3 m*M*. In the case of 5 m*M* isoproterenol, a beta agonist, Thorpe et al found that the H^+ secretory rate was significantly decreased by 55% and 60% respectively for histamine and acetylcholine-stimulated H^+ secretion. This inhibition of isoproterenol was blocked by propranolol, a beta antagonist. The authors suggested on the basis of their data that isoproterenol inhibited acid secretion by direct activation of mucosal beta-adrenergic receptors. Recent work in our laboratory (5) lends support to their experimental findings on beta adrenergic amines.

In the study of Thorpe *et al.* (3), however, only the H^+ secretory rate was measured. The present investigation considers the inhibitory effects of isoproterenol (Isuprel), a beta agonist; epinephrine (Adrenalin) a predominantly beta agonist; and propranolol (Inderal), a beta antagonist on the transmucosal resistance, the transmucosal potential difference (PD) and the H^+ secretory rate of the gastric mucosa of Rana pipiens in Cl^- media. In addition, blocking effects between propranolol and isoproterenol and between propranolol and epinephrine are reported.

Methods. The experiments were performed on gastric mucosae of Rana pipiens with an in vitro method described in detail elsewhere (6). Two pairs of electrodes were used, one for sending current across the mucosa and the other for measuring the PD. The resistance was obtained as the change in PD per unit of applied current. The H^+ secretory rate was determined by the pH stat method introduced by Durbin and Heinz (7). The pH of the secretory side was maintained at 4.90. The nutrient bathing solution contained (in m*M*): Na^+, 102; K^+, 4; Ca^{2+}, 1; Mg^{2+}, 0.8; Cl^-, 81; HCO_3^-, 25; phosphate, 1.0; and glucose, 10; and the secretory bathing solution contained: Na^+, 102; K^+, 4; Cl^-, 106. Both sides of the mucosa were gassed with 95% O_2 and 5% CO_2. Histamine was added to the nutrient solution to a concentration of 10^{-4} *M*, thereby providing maximal stimulation of the gastric mucosa. After the control part of the experiment, the beta adrenergic amine was added to the nutrient solution in concentrations varying from 0.1 m*M* up to as much as 4.7 m*M*. Combinations of these amines were also investigated. For each experiment a different frog was used.

Results. Electrophysiological effects of varying concentrations of beta adrenergic amines. Figure 1 depicts the results of a typical experiment in which the nutrient solution contained isoproterenol at a concentration of 0.25 m*M*. In 30 min after the

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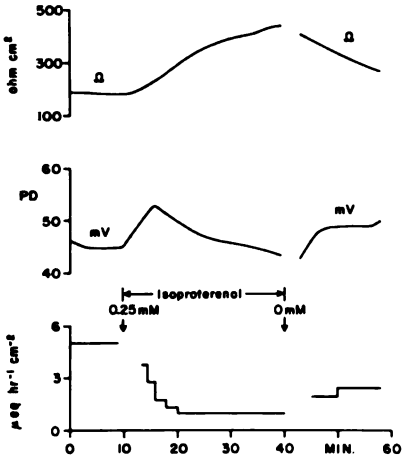

FIG. 1. Effects of isoproterenol on the resistance, PD and H^+ secretory rate of frog gastric mucosa in chloride solutions. At the time indicated by the first arrow, isoproterenol was added to a concentration of 0.25 m*M* in the nutrient solution and at the time indicated by the second arrow, the isoproterenol was removed. Resistance, PD and H^+ secretory rate are plotted versus time.

addition of isoproterenol, the resistance increased about 100% above control values while the H^+ secretory rate decreased about 80%. The PD at first increased to a maximum value but at the end of 30 min returned essentially to the control level. Washing both sides of the mucosa with regular solutions produced a partial recovery of the resistance and H^+ secretory rate towards control levels.

Table I summarizes the results of the effects induced by each of the three adrenergic amines about 30 min after the addition of a specified concentration of the amine to the nutrient solution, compared to control values. Except for 0.1 m*M* isoproterenol and 0.1 m*M* propranolol, all concentrations of each amine gave significant increases in resistance. Without exception, each amine showed significant decreases in the H^+ secretory rate at all concentrations. In fact, there was a dose dependency in that for each amine the H^+ secretory rate became increasingly smaller with increasing concen-

TABLE I. EFFECTS OF BETA ADRENERGIC AMINES ON RESISTANCE, PD AND H^+ SECRETORY RATE OF FROG GASTRIC MUCOSA.

Adrenergic amines	Conc. (m*M*)	No. of Expts.	R (Ω-cm^2)	$\Delta R/R$ (%)	PD (mV)	$\Delta PD/PD$ (%)	$\dot{H}$ (μeq h^{-1} cm^{-2})	$\Delta\dot{H}/\dot{H}$ (%)
Isoproterenol	0.10	6	183 ± 67[a]	8 ± 14 ($P > 0.1$)	34 ± 5	14 ± 6 ($P < 0.01$)	6.0 ± 2.1	−9.7 ± 5.8 ($P < 0.05$)
	0.25	9	193 ± 59	111 ± 77 ($P < 0.01$)	37 ± 8	12 ± 10 ($P < 0.01$)	4.2 ± 1.0	−62.0 ± 24 ($P < 0.01$)
	0.50	6	237 ± 101	279 ± 172 ($P < 0.01$)	42 ± 3	−6 ± 15 ($P > 0.4$)	4.1 ± 0.7	−100 ± 0 ($P < 0.01$)
Epinephrine	0.10	7	336 ± 123	17 ± 11 ($P < 0.01$)	44 ± 11	6 ± 9 ($P > 0.20$)	3.1 ± 0.7	−22 ± 12 ($P < 0.01$)
	1.00	14	203 ± 91	122 ± 112 ($P < 0.01$)	28 ± 8	32 ± 20 ($P < 0.01$)	4.6 ± 1.1	−70 ± 31 ($P < 0.01$)
	2.00	6	313 ± 130	69 ± 49 ($P < 0.02$)	34 ± 5	14 ± 8 ($P < 0.01$)	3.3 ± 1.1	−78 ± 35 ($P < 0.01$)
	4.70	3	127 ± 29	725 ± 141 ($P < 0.01$)	46 ± 6	−4 ± 16 ($P > 0.7$)	4.7 ± 0.7	−100 ± 0 ($P < 0.01$)
Propranolol	0.10	8	190 ± 101	5.1 ± 9.9 ($P > 0.4$)	30 ± 7	−4 ± 16 ($P > 0.5$)	3.6 ± 1.0	−13 ± 10.8 ($P < 0.01$)
	1.00	7	303 ± 99	19 ± 14 ($P < 0.02$)	41 ± 8	−11 ± 8 ($P < 0.02$)	2.8 ± 0.8	−28 ± 26 ($P < 0.05$)
	2.00	7	332 ± 166	47 ± 31 ($P < 0.01$)	35 ± 9	−12 ± 9 ($P < 0.05$)	3.0 ± 1.4	−51 ± 25 ($P < 0.05$)

[a] Values are means ± SD. $\dot{H}$ refers to H^+ secretory rate. R, PD, and $\dot{H}$ refer to nutrient solution without amine (control values). $\Delta R/R$, $\Delta PD/PD$, and $\Delta\dot{H}/\dot{H}$ refer to relative changes produced 30 min after the addition of the amine to the nutrient solution. Statistics were determined from differences and not from percentages changes.

tration of that amine in the nutrient solution.

In contrast, changes in PD showed some specificity in that the effects of the beta antagonist and the beta agonists differed with increasing concentrations. We note that for propranolol the PD changed insignificantly for 0.10 m*M* concentration but decreased significantly for the higher concentrations, thereby showing the same PD changes as for the alpha adrenergic amines (4). Contrary to this, the beta agonist, isoproterenol, showed an increase in PD for the lower concentrations of 0.10 and 0.25 m*M* and an insignificant change for the highest concentration of 0.50 m*M*. With the exception of 0.10 mM concentration of epinephrine resulting in an insignificant change in PD, the higher concentrations of epinephrine showed the same trend as isoproterenol. It was this difference in the change in PD between agonist and antagonist that suggested the possibility of blocking effects.

Effects of a combination of agonist and antagonist in the nutrient solution following an induction period with a single amine. Figure 2 illustrates a typical experiment in which 0.10 m*M* propranolol in the nutrient solution for 30 min was followed by a combination of 0.10 m*M* propranolol and 0.25 m*M* isoproterenol. As Fig. 2 shows, the changes in the parameters resulting from the combination of amines after 30 min in the nutrient solution were only minimal compared to control levels. The resistance increased by about 15%, the PD decreased by about 20% and the H^+ secretory rate decreased by about 15%. A comparison of Fig. 2 with Fig. 1 shows that isoproterenol alone produced marked changes in resistance and H^+ secretory rate compared to small changes by the combination of amines. Finally, there was a recovery period with regular solutions in which the parameters returned towards control levels.

Table II summarizes the effects induced by a combination of adrenergic amines after a period of 30 min in the nutrient solution following a single amine in that solution for 30 min. In experiments with 0.10 m*M* propranolol followed by a combination of 0.10 m*M* propranolol and 0.25 m*M* isoprotere-

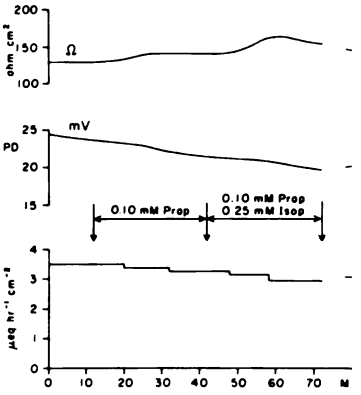

FIG. 2. Effects of propranolol (Prop) a terenol (Isop) following an induction period pranolol alone on the resistance, PD and H^+ rate of frog gastric mucosa in chloride sol the time indicated by the first arrow, propr added to a concentration of 0.10 m*M* in th solution. At the time indicated by the sec 30 min later, isoproterenol was added to a tion of 0.25 m*M* in the nutrient solution had the combination of two amines. At indicated by the third arrow, the two am removed.

nol, the effects of the combination c ance, PD and H^+ secretory rate wer cantly less compared to the effects m*M* isoproterenol alone. We note 25% decrease in H^+ secretory rat the combination is significantly sm < 0.01) than the 62% decrease secretory rate due to isoprotereno Also the insignificant change in re and PD due to the combination c significantly ($P < 0.01$) with th increase in resistance and the 12% in PD due to isoproterenol alone. H if 0.25 m*M* isoproterenol alone in t ent solution was followed by the c tion of 0.25 m*M* isoproterenol a m*M* propranolol, the effects obta the combination after 30 min in the solution on resistance, PD and H^+ s rate were not significantly differe the effects on these parameters obt isoproterenol alone.

As Table II indicates, the combir 0.1 m*M* propranolol and 1.0 m*M* rine exhibited the same kind of be the combination of 0.1 m*M* pro and 0.25 m*M* isoproterenol. If pro

TABLE II. EFFECTS OF COMBINATION OF TWO ADRENERGIC AMINES ON RESISTANCE, PD AND H^+ SECRETORY RATE OF FROG GASTRIC MUCOSA FOLLOWING AN INDUCTION PERIOD WITH A SINGLE AMINE.[a]

Combination of Adrenergic Amines	No. of Expts.	R (Ω-cm^2)	$\Delta R/R$ (%)	PD (mV)	$\Delta PD/PD$ (%)	$\dot{H}$ (μeq h^{-1} cm^{-2})	$\Delta\dot{H}/\dot{H}$ (%)
0.10 m*M* Propranolol[a]	9	179 ± 100[b]	24 ± 32	29 ± 7	−10 ± 21	2.9 ± 1.4	−25 ± 26
0.25 m*M* Isoproterenol			($P > 0.1$)		($P > 0.1$)		($P < 0.01$)
0.25 m*M* Isoproterenol	6	248 ± 126	66 ± 46	25 ± 9	16 ± 163	3.5 ± 0.9	−73 ± 26
0.10 m*M* Propranolol			($P < 0.01$)		($P > 0.50$)		($P < 0.01$)
0.10 m*M* Propranolol	7	216 ± 118	42 ± 28	23 ± 12	42 ± 58	4.0 ± 2.0	−30 ± 22
1.00 m*M* Epinephrine			($P < 0.01$)		($P > 0.05$)		($P < 0.01$)
1.00 m*M* Epinephrine	6	181 ± 93	117 ± 93	26 ± 9	27 ± 15	4.1 ± 0.8	−66 ± 27
0.10 m*M* Propranolol			($P < 0.05$)		($P < 0.01$)		($P < 0.01$)
0.10 m*M* Epinephrine	4	271 ± 161	44 ± 21	38 ± 11	5 ± 14	3.2 ± 0.8	−65 ± 12
1.00 m*M* Propranolol			($P < 0.05$)		($P > 0.20$)		($P < 0.01$)
0.10 m*M* Propranolol	4	341 ± 122	77 ± 47	40 ± 6	25 ± 11	3.4 ± 0.9	−85 ± 13
1.00 m*M* Norepinephrine			($P < 0.02$)		($P < 0.02$)		($P < 0.02$)

[a] First member of combination was single amine in nutrient solution to which second member of pair was later added.

[b] Values are means ± SD. $\dot{H}$ refers to H^+ secretory rate. R, PD, and $\dot{H}$ refer to nutrient solution without amine. $\Delta R/R$, $\Delta PD/PD$, and $\Delta\dot{H}/\dot{H}$ refer to relative changes produced 30 min after the addition of the combination of amines in the nutrient solution following a 30-min period of a single amine in the solution.

was added first, blocking effects were evident whereas if epinephrine was added first, blocking effects were absent. All other combinations failed to reveal any antagonism. For example, previously it was found that the alpha agonist, norepinephrine, and the alpha antagonist, phentolamine, showed no blocking effects (4). From Table II, it is evident that there is no interference between the beta antagonist, propranolol, and the alpha agonist, norepinephrine.

Discussion. Agents or procedures which markedly increase the resistance of the H^+- and/or Cl^- active pumps on the secretory membrane produce a concurrent marked decrease in the H^+ secretory rate (8, 9). On the other hand, agents or procedures which increase the resistance of ionic pathways on the nutrient membrane are usually associated with small decreases in the H^+ secretory rate (9, 10). Rose *et al.* (4) provided an analysis based on these facts to explain changes in PD arising from changes in resistance. We shall apply this analysis as a working model to the present results. Referring to Fig. 3 which shows the principal ionic pathways on the secretory and nutrient membranes, we shall summarize the main results of the analysis at this point.

When any treatment results in an increase in resistance and a marked decrease in H^+ secretory rate, it is, as indicated above,

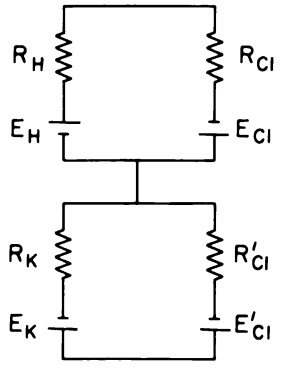

FIG. 3. Electrical circuit showing principal pathways of the secretory and nutrient membranes. Upper part refers to pathways of the secretory membrane, namely the H^+ and Cl^- emf's and their respective resistances R_H and R_{Cl}. Lower part refers to low resistance pathways of the nutrient membrane, namely the emf's E_K and E'_{Cl} due to ion gradients and their respective resistances R_K and R'_{Cl}.

reasonable to assume its effect to be mainly on the secretory membrane. Should changes in R_H by ΔR_H and R_{Cl} by ΔR_{Cl} both be involved, the changes in PD would be as follows. The PD decreases if $(R_{Cl} + \Delta R_{Cl})/(R_H + \Delta R_H) > R_{Cl}/R_H$, that is, if R_{Cl} has a higher proportional increase than R_H. The PD increases if R_H increases proportionally more than R_{Cl}. Finally, the PD does not change if both R_{Cl} and R_H have equal

proportional increases.

When any treatment results in increases in resistance and small changes in H^+ secretory rate, it is reasonable to assume that the effect is mainly on the nutrient membrane. The interpretation here is complicated by the fact that the relative magnitudes of E'_{Cl} and E_K are not well established. If one assumes that $E'_{Cl} > E_K$ (10), the PD decreases if $(R'_{Cl} + \Delta R'_{Cl})/(R_K + \Delta R_K) > R'_{Cl}/R_K$, that is, if R'_{Cl} increases proportionally more than R_K. The PD increases if the reverse is true. Should it be shown that $E_K > E'_{Cl}$, opposite effects would be obtained. The PD does not change if both R'_{Cl} and R_K increase proportionally the same.

The effects of the adrenergic amines on the ionic pathways can now be explained. For those concentrations in which the beta agonist, isoproterenol or epinephrine, shows both an increase in resistance and an increase in PD, it may be inferred that the principal effects must occur on the H^+ pathway of the secretory membrane and on the K^+ pathway of the nutrient membrane (if $E'_{Cl} > E_K$). On this basis, the Cl^- pathway could be affected on each membrane but to a lesser extent than the cationic pathways. At the highest concentrations of both amines at which secretion was abolished, there occurred an increase in resistance and no significant change in PD. These results imply that the H^+ and Cl^- pathways of the secretory membrane change proportionately about the same. For those concentrations in which the beta antagonist, propranolol, shows both an increase in resistance and a decrease in PD, it may, likewise, be inferred that the principal effects may occur on the Cl^- pathways of both membranes (if $E'_{Cl} > E_K$). The effects of propranolol on the PD and resistance compare with those of norepinephrine and phentolamine (4).

We shall now discuss briefly the blocking effects. Even though the concentrations were much higher than those needed in other tissues for studies of alpha and beta receptors, it is of interest that even at these high concentrations there seems to be a type of antagonism. Except for the large concentrations, the data are compatible with the concept of beta receptor sites as hypothesized by Thorpe *et al.* One might speculate that isoproterenol or epinephrine would inhibit primarily at these sites whereas propranolol would also act at non-specific sites. Thus neither isoproterenol nor epinephrine would interfere substantially with propranolol but the latter at the beta receptor sites would prevent the inhibitory action of isoproterenol or epinephrine. The existence of such sites needs to be established using the criteria for competitive antagonism and demonstrating affinity of the receptors as comparable with known beta receptors.

Summary. Beta adrenergic amines like alpha adrenergic amines decreased the H^+ secretory rate and increased the transmucosal resistance of the gastric mucosa of Rana pipiens. A concentration of 0.1 m*M* propranolol in the nutrient solution followed by a combination of 0.1 m*M* propranolol and either 0.25 m*M* isoproterenol or 1.0 m*M* epinephrine reduced the effects of the beta agonists, isoproterenol and epinephrine. However, a concentration of 0.25 m*M* isoproterenol or 1.0 m*M* epinephrine followed by the same combination did not reduce the effects of isoproterenol or epinephrine alone. These results suggested the existence of beta receptor sites in addition to non-specific sites.

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Elevated Thymidine Phosphorylase Activity in the Plasma and Ascitic Fluids of Tumor-Bearing Animals[1] (40034)

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Neoplasia is often characterized by a qualitative and quantitative imbalance of key enzymes, particularly those regulating DNA and RNA synthesis (1, 2). Having defined more fully those metabolic alterations associated with tumors of a particular histopathology, these key enzymes will provide useful biomarkers for identifying and monitoring tumor progression, and will continue to be the major targets of different chemotherapy regimens (1).

We have recently identified thymidine (TdR) phosphorylase (EC 2.4.2.4; TdR: orthophosphate deoxyribosyltransferase) in the plasma of man and different laboratory animals (3), and have shown that patients with uncontrolled neoplastic diseases often exhibit levels of this pyrimidine nucleosidase that are significantly greater than those of healthy subjects (4). This enzyme catalyses the reversible reaction of TdR + orthophosphate $\rightleftharpoons$ thymine (Thy) + 2-deoxy-α-D-ribose-1-phosphate (5), and its presence in many normal and neoplastic cells of man and different animals as well as in various pathogenic and nonpathogenic prokaryotic cells has been well documented (5, 6). Some investigators have suggested that, under certain circumstances, this enzyme may play an important role in the salvage of Thy for nucleic acid synthesis in tissues having a high rate of proliferation; however, its physiological function, especially with respect to malignant tissues, has not been fully elucidated.

To further define the potential clinical and experimental utility of assaying TdR phosphorylase activity, a study of different tumor-bearing animals was undertaken. Reported herein are the results of experiments demonstrating that significant increases in this TdR-degrading enzyme were recorded in most of the tumor models tested.

Materials and methods. Adult rats and mice of different strains were used, and initial experiments were performed using animals that had been divided into 11 groups (Groups A-K, Table I). Each of these groups consisted of test animals having a tumor of a particular histological type and about an equal number of age- and sex-matched healthy control animals. The pathophysiology of these tumors and procedures used for their serial passage have been described elsewhere (7-13, see Table I).

Plasma was from fresh blood that had been collected asceptically into siliconized tubes containing EDTA (1.5 mg/ml) and sodium heparin (10 units/ml). Rat blood was collected by retroocular puncture and mouse blood was procured by decapitation. Ample blood from a single animal was obtained such that replicate enzyme determinations could be made using unpooled plasma samples. Ascitic fluid was aspirated from the peritoneal cavity with a syringe and needle. After performing a cell count, the fluid was centrifuged (250g, 8 min) and the resulting pellet containing tumor cells was washed twice with phosphate buffered saline (PBS, pH 7.2; 0.14 M NaCl, 4.0 mM KCl, 0.5 mM Na_2HPO_4 and 0.15 mM KH_2PO_4). Thereafter, the cells were resuspended to their original density in PBS and then disrupted by ultrasonication. With the exception of the mouse plasma, all samples to be assayed were passed through a micropore (0.22 μm) filter.

TdR phosphorylase activity was measured using our microassay (14); this is a modification of techniques that have been de-

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TABLE I. THYMIDINE PHOSPHORYLASE ACTIVITY IN THE PLASMA OF HEALTHY AND TUMOR-BEARING ANIMALS.

Group	Species (strain)	Tumor model	Animals assayed	No. of animals	% [^{3}H]TdR degraded (mean ± SE)	Significance (Student's *t* test)
A	Rat (Furth-Wistar)	Wilms' tumor (7)[a]	Healthy control	8	1.9 ± 0.3	$P < 0.001$
			Tumor-bearing	8	9.1 ± 0.6	
B	Rat (Charles River CD)	Zajdela ascites hepatoma (8)	Healthy control	8	2.6 ± 0.5	$P < 0.001$
			Tumor-bearing	8	17.5 ± 3.5	
C	Mouse (DBA/2)	L1210 lymphoid leukemia (9)	Healthy control	8	1.5 ± 0.3	$P < 0.001$
			Tumor-bearing	22	55.9 ± 7.2	
D	Mouse (RFM/Un)	Myeloid leukemia (10)	Healthy control	8	3.3 ± 0.4	$P < 0.001$
			Tumor-bearing	8	52.6 ± 4.0	
E	Mouse (A/Ha)	L#2 ascites lymphoma (11)	Healthy control	5	2.5 ± 0.5	$P < 0.01$
			Tumor-bearing	5	48.9 ± 13.2	
F	Mouse (Ha/ICR)	Ehrlich ascites tumor (9)	Healthy control	5	2.9 ± 0.9	$P < 0.05$
			Tumor-bearing	9	16.8 ± 1.6	
G	Mouse (Balb/c)	Neuroblastoma (9)	Healthy control	5	2.1 ± 0.2	$P < 0.005$
			Tumor-bearing	5	16.7 ± 3.3	
H	Mouse (BDF_1)	B16 melanotic melanoma (12)	Healthy control	6	2.3 ± 0.3	$P < 0.01$
			Tumor-bearing	6	8.9 ± 1.9	
I	Mouse (C3H/Bi)	Renal cell carcinoma (9)	Healthy control	9	2.3 ± 0.2	$P < 0.025$
			Tumor-bearing	9	6.7 ± 1.5	
J	Mouse (ICR × C57Bl/6Ha)	Gyn-74 uterine tumor (NR)[b]	Healthy control	10	3.7 ± 0.8	$P > 0.1$
			Tumor-bearing	11	5.4 ± 0.9	
K	Mouse (DBA/2)	Friend erythroblastic leukemia (13)	Healthy control	5	5.4 ± 1.3	$P > 0.4$
			Tumor-bearing	5	3.9 ± 1.2	

[a] Literature reference.
[b] Not reported.

scribed previously (15–17). In brief, 20 μl of plasma, ascitic fluid or sonic extract was added to a 200 μl U-shaped culture-well of a microtiter plate. To each culture-well was then added 20 μl of PBS containing 1.0 μCi [6-^{3}H] TdR (spec. act., 2.0 Ci/mmole; final conc., 12 m*M*). Immediately after mixing (time 0 hr, background samples) and after incubating at 37° for various times (usually 3.0 hr, test samples), 5 μl of the plasma–isotope mixture was spotted onto a Whatman No. 1 chromatography paper at a location that had been spotted previously with 5 μl of a saturated mixture of unlabeled TdR and Thy reference markers. Chromatography was performed as described previously (14–17) and radioactivity in grid sections containing the uv-absorbing TdR and Thy reference standards of each chromatography channel was then determined by liquid scintillation counting ($\cong 1 \times 10^5$ cpm; counting efficiency, 45 ± 0.1%). After subtracting background [^{3}H] Thy values (<3%), the amount of [^{3}H]TdR or [^{3}H]Thy present in each sample was defined as a percentage of the total radioactivity (amount recovered, ≥95%). Human plasma from patients showing high levels of TdR phosphorylase activity (4) were often included as standards for monitoring technical variability. These included aliquots of plasma which had (negative standard) or had not (positive standard) been heated (56°, 2 hr) to inactivate this enzyme before storing at −20°. Reproducibility among replicate assays was good (mean S.E., ≤5%). Enzyme activity was expressed as the percent [^{3}H]TdR degraded; this departure from conventional definitions of enzyme activity (i.e., product formation rather than substrate degradation) was justified in that it illustrated the potential magnitude of error that may arise in various *in vitro* or *in vivo* assays in which the cellular uptake of [^{3}H]- or [^{14}C]TdR is used as the sole basis for defining DNA synthesis (3, 4, 14–17).

TdR kinase activity was assayed using the method described by Lee and Cheng (18). The reaction mixture contained 0.19 *M* Tris-HCl (pH 7.5), 1.9 m*M* $MgCl_2$, 0.2 μCi [^{14}C]TdR (spec. act., 12 μCi/mmole; final conc., 0.19 m*M*), 1% bovine serum albumin, 3 mM phosphocreatine, 0.54 units creatinine kinase, 10 m*M* dithiothreitol, 10 m*M* NaF and test serum in a final volume of 0.1 ml. The assay mixture was usually incubated for 1.0–2.0 hr; however, in some assays, longer times were tested. The reaction was stopped by spotting 50 μl of the reaction mixture onto a Whatman DE81 disc, then immediately dropping it into alcohol (10 ml/disc) and washing three times with alcohol. Thereafter, the sample was prepared for liquid scintillation counting and the amount of [^{14}C]TMP formed was determined.

The results were subjected to statistical evaluation by means of the Student's *t* test for small samples, and differences between means giving a probability of less than 5% were considered to be significant.

Results. Summarized in Table I are the results of a survey defining TdR phosphorylase activity in the plasma of healthy and tumor-bearing rats ($N = 32$) and mice ($N = 131$). Significant ($P < 0.01$) differences in TdR phosphorylase activity were recorded for each of the two rat tumor models; in these experiments, enzyme activity in the plasma from animals with Wilms' tumor or Zajdela ascites hepatoma (Groups A and B) were 379 and 573% greater than the corresponding values for animals without tumors.

Significantly ($P < 0.05$) elevated TdR phosphorylase activity was also identified in 7 of the 9 (78%) murine tumor models examined. Of these, highest levels of enzyme activity were recorded for animals with lymphoid leukemia (Group C), myeloid leukemia (Group D) and ascites lymphoma (Group E). Increased activity was also recorded for mice with Ehrlich ascites tumor (Group F).

Of the four test groups with different types of solid tumors (Groups G–J), increased plasma TdR phosphorylase activity was noted in three (75%). Test mice failing to show an increase in TdR phosphorylase activity were those with a carcinogen-induced uterine tumor. This uterine tumor, which is currently being characterized, was transplanted subcutaneously and, when compared with other tumors in this study, was rather benign in that it showed little or no evidence of metastasis. Mice with Friend erythroblastic leukemia (Group K) also failed to show an increase in plasma TdR phosphorylase activity.

Presented in Table II are the results of a study comparing TdR phosphorylase activity in the plasma, ascitic fluids and tumor cell sonicates of mice with L1210 lymphocytic leukemia. This leukemia was transplanted intraperitoneally with 1.5×10^6 tumor cells and the animals were sacrificed 9 days later. Appreciable TdR phosphorylase activity was recorded in 0.5 hr reaction mixtures. Increased amounts of [^{3}H]TdR were degraded in reaction mixtures of 1.0–4.0 hr; mean values (±S.E., $N = 9$ mice) obtained in 2.0 hr reaction mixtures containing tumor cell sonicates, plasma and ascitic fluids were 45.0 ± 6.5, 31.8 ± 11.5 and 6.5 ± 1.6%, respectively.

In similar studies, corresponding mean values recorded in 3.0 hr assays of ascitic fluids from rats with Zajdela hepatoma (*N*

TABLE II. THYMIDINE PHOSPHORYLASE ACTIVITY IN THE PLASMA, ASCITIC FLUIDS AND TUMOR CELL SONICATES OF MICE WITH L1210 LYMPHOCYTIC LEUKEMIA.

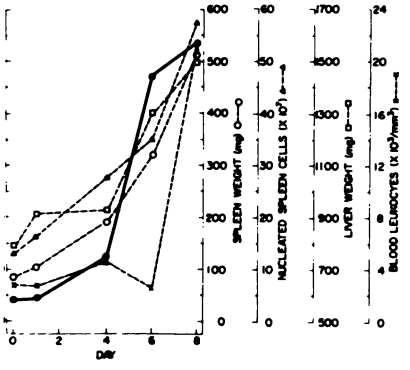
Substance assayed	% [^{3}H]TdR degraded in reaction mixtures of:			
	0.5 hr	1.0 hr	2.0 hr	4.0 hr
Plasma	19.4 ± 9.5[a]	26.0 ± 11.4	31.8 ± 11.5	40.1 ± 11.4
Cell sonicates[b]	15.6 ± 2.2	27.5 ± 3.4	45.0 ± 6.5	61.6 ± 5.1
Ascitic fluids	3.2 ± 0.7	5.3 ± 1.7	6.5 ± 1.6	11.5 ± 2.6
Ascitic fluids-H[c]	0.3 ± 0.1	0.3 ± 0.1	0.8 ± 0.4	1.3 ± 0.6

[a] Mean ± SE, $N = 9$ mice.
[b] Cell density (mean ± SE) before lysis, $12.7 \pm 0.6 \times 10^7$/ml PBS.
[c] Heated (H) at 56° for 2.0 hr.

mice with L#2 lymphoma and mice
ırlich tumor (N = 13) were 19.2 ±
.3 ± 10.1 and 29.2 ± 5.9, respec-

:nted in Fig. 1 are the results of a
:omparing changes in plasma TdR
orylase activity with tumor progres-
mice with myeloid leukemia. Param-
f tumor growth which were moni-
ıcluded changes in the spleen weight,
ed spleen cells, liver weight and
eukocytes. Enzyme activity and these
ıt parameters were analyzed for mice
anted intravenously with 1.0 × 10^6
leukocytes from a leukemic mouse.
ȝ of animals (N = 3/group) were
ed on days 0 (i.e., immediately after
transfer), 1, 4 and 8. There were no
changes within the first day. On day
/ever, we detected a significant in-
in TdR phosphorylase activity that
companied by splenomegaly. These
s, as well as the concomitant devel-
t of hepatomegaly, became more
ınced on day 6. On day 8, a time at
the mice were in the terminal phase
ir disease, highest values were re-
for TdR phoshorylase activity and
h of the four morphological parame-

vn in Fig. 2 are the results of a
:ntative study of TdR kinase and TdR



1. Comparison of different morphological pa-
of tumor progression with thymidine phos-
e activity in the plasma of mice having myeloid
ı. Assays were performed immediately after
ransplantation (day 0) and at various times
:r. Each point denotes the mean value of three
Reproducibility of the different assays was
ually less than 15% SE.

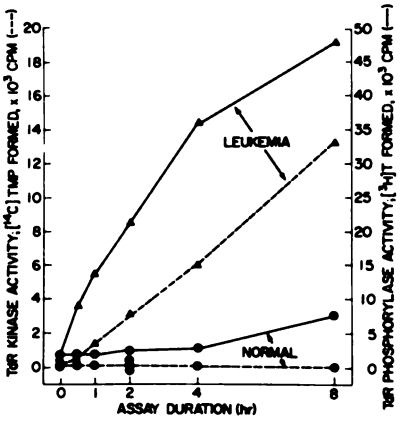


FIG. 2. Analysis of TdR kinase and TdR phosphorylase activities in the sera of a test mouse in the terminal stage of his myeloid leukemia and of a corresponding healthy control animal. Each point denotes the mean value of triplicate assays, SE ≤ 7%.

phosphorylase activities in the sera of a moribund mouse with myeloid leukemia and of a healthy animal of the same strain, age and sex. Significant (P < 0.001) increases in both of these enzymes were recorded for the leukemic mouse, and activities of these enzymes were nearly linear in 4.0 hr reactions. Values for the corresponding enzymes in the sera of the normal mouse were consistently low.

Discussion. Selected for initial experimentation were animals having tumors that differed markedly with respect to their strain specificity, etiology, histology, growth, invasiveness and morbidity. Results of these studies demonstrated that TdR phosphorylase activity in the plasma of tumor-bearing animals was increased significantly in 9 of 11 (82%) models tested.

The findings of experiments reported herein are consistent with the observations of our previous report showing that the activity of this TdR-degrading enzyme is frequently elevated in the plasma of patients with uncontrolled neoplastic diseases who had or had not received antitumor therapy (4). Accordingly, increased plasma TdR phosphorylase activity in the tumor-bearing patient cannot be ascribed solely to impaired cell function resulting from chemotherapy, radiotherapy, surgery or other antitumor modalities. Moreover, it is unlikely that elevated activity of this extracellular

enzyme can be attributed to undetected viral, bacterial or fungal infections. Thus, changes in TdR phosphorylase activity noted in these tumor-bearing hosts are thought to be associated with tumor growth. Equally important, however, is that studies reported herein have identified several tumor models suitable for future experiments characterizing the origin, function and kinetics of this enzyme.

The reasons for this increased activity of TdR phosphorylase are not known, and we cannot exclude as possible explanations the release of TdR by rapidly replicating tumor cells, by tumor tissue necrosis and disoluton, or by normal tissues whose function has been altered either directly by metastatic infiltration or indirectly by humoral factors.

In previous studies attempting to define the mechanisms whereby TdR phosphorylase is released, we have shown that high levels of this enzyme are elaborated by lymphoblastoid cells of established human lines when these cells were maintained for a short time under certain culture conditions having no apparent adverse affect on their viability or morphology (14). Other *in vitro* experiments have shown that TdR phosphorylase is also released by peripheral blood leukocytes of healthy donors as well as of patients with either myeloid or lymphoid leukemia (14-19). These and other studies currently in progress suggest that the extracellular presence of TdR phosphorylase is not merely a consequence of cell death, and that it may be selectively released as are the lysosomal enzymes of polymorphonuclear leukocytes (21). In this respect it is noteworthy that the polymorphonuclear leukocyte is thought to contain significantly higher levels of TdR phosphorylase than mononuclear leukocytes (15-17).

The biochemical strategy of cancer cells, particularly with respect to the imbalance of enzymes of pyrimidine synthesis and degradation and in the *de novo* and salvage pathways of DNA, have been studied extensively. Of particular interest have been the findings of Weber and co-workers who have formulated the "molecular-correlation concept" (1, 2). Essential to this idea is the existence of certain enzymes whose concentrations and activities increase, and those of opposing enzymes that decreased, with malignant transformation or malignant progression (1, 2). Examination of homogenates or sonic extracts of different types of tumors as well as of tumors having a similar histology but varied growth rates has provided evidence suggesting that intracellular TdR phosphorylase activity is decreased in most tumors during periods of accelerated growth. The reason for the apparent discrepancy between the activity of intracellular and extracellular TdR phosphorylase activity cannot readily be resolved with data that are currently available.

Assuming that the "molecular-correlation concept" is correct, an interpretation of our findings of increased TdR phosphorylase activity in the tumor-bearing host is further complicated by information from recent publications reporting increased tumor-associated TdR kinase activity in the sera of rats with transplantable hepatomas (19) and lymphomas (20), but not of hosts carrying hepatic (observations conflict with those of reference 19), renal, mammary or submaxillary gland tumors (20). In addition to the studies of mice with myeloid leukemia (see Fig. 2), we have also observed increased TdR kinase activity in the sera of other animals with transplanted tumors including rats with hepatomas (unpublished). This apparent biochemical imbalance of TdR phosphorylase and TdR kinase, enzymes of the pyrimidine pathway that are functionally opposed to one another (1, 2), requires further characterization in serial studies correlating the activities and concentrations of these enzymes in both tumor cells and plasma with tumor progression and the pathophysiology of the host.

Summary. Activity of thymidine (TdR) phosphorylase (EC 2.4.2.4; TdR: orthophosphate deoxyribosyltransferase) in the plasma of healthy and tumor-bearing mice and rats was evaluated in comparative studies. Increased enzyme activity was recorded in 9 of 11 tumor models tested, and highest values were obtained for mice with lymphoid or myeloid leukemia. Heightened activity of this TdR-degrading enzyme was observed in the plasma of mice bearing three of four solid tumors. Elevated TdR phos-

)horylase activity was also identified in the ieritoneal exudate fluids in each of four roups of animals carrying ascites-producing umors.

Tumor-bearing animals used in these studies were indly provided by investigators at Roswell Park Memrial Institute, including Drs. J. T. Evans, T. S. auschka, M. T. McGarry, G. P. Murphy, H. D. riesler and E. J. Sarcione.

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Radioimmunoassay of Measles Virus Antigen and Antibody in SSPE Brain Tis (40035)

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An indirect solid phase radioimmunoassay (RIA) method developed in this laboratory is highly sensitive for detecting viral antigens in cell cultures and brain tissue, and for assay of viral antibodies in sera and cerebrospinal fluids (1-4). In the present study, the RIA was applied to detection of measles virus antigen in brain tissue of experimentally infected animals and of a patient with subacute sclerosing panencephalitis (SSPE); for detecting specific measles virus antibody in eluates from SSPE brain tissue; and for determining the classes of immunoglobulins represented in the eluted antibody. It was found that RIA could be applied to detection of both measles virus antigen and antibody in the same brain tissue. Elution of brain tissue with chaotropic ions (5) was shown to release greater amounts of specific viral antibody than did elution at an acid pH.

MATERIALS AND METHODS. *Measles virus-infected cell cultures.* The LEC strain of measles virus, isolated from the brain of a child with SSPE (6), was obtained from Dr. H. Koprowski, Wistar Institute. Human fetal diploid lung (HFDL) cells propagated in the bottom of 1-dram glass vials were infected with the virus at a multiplicity of 1, and these were used, as previously described (4), as a source of antigen for assay of measles virus antibody by RIA. Uninfected cells prepared in the same manner were used as controls.

For studies on the adsorption and elution of measles virus antibody to and from infected cells in culture, HFDL cell monolayers in roller bottles were infected with LEC virus at a multiplicity of 1, incubated for 5 or 6 days at 36°. The cells were then dislodged from the bottle surface by sh with glass beads, sedimented by centri tion at 2000 rpm for 5 min, washed with phosphate buffered saline, pH (PBS), and used for the adsorption-el studies described below.

Measles virus-infected brain tissue. LEC strain of measles virus adapte growth in suckling hamster brains was ulated by the intracerebral route into sters 2 to 3 days of age using an inoc of 0.02 ml containing approximately 10^3-1 × 10^4 pfu of virus. After 3 to 4 when the animals first showed signs ness, the brains were harvested and s at −70°.

The human brain tissue studied was a child with SSPE. Upon initial examin in our diagnostic laboratory the showed positive fluorescent antibody staining with measles antiserum. It had stored at −70° for 8 years at the time present study.

Frozen sections from the brain tissue mounted onto 15 mm round glass c slips, using five sections per coverlsip out overlapping, and the tissue was air- and fixed in cold acetone for 10 min. coverslips were then transferred to scr tion vials for use in RIA. Adjacent sec from the tissue were mounted on glass for FA staining, air-dried, and fixed acetone.

Measles virus antiserum. Hamsters immunized with the HNT Philade strain 26 neurotropic virus (7) to pr measles antiserum. This was used intermediate serum in the RIA e ments, and also for indirect FA stainir

^{125}I-labeled antibodies to human and ster immunoglobulins. Heavy chain-sp antisera to human IgA, IgG and IgM duced in goats were obtained from stad Laboratories, Chaska, MN, and

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m to hamster gamma globulin from lies, Inc., Davis, CA. These were using immunoadsorbents prepared urified homologous immunoglobu-

n IgA was isolated and purified 0 ml of pooled human serum by the of Newcomb *et al.* (8). The concen- gA was chromatographed twice on 00 cm columns of Sephadex G-200, fractions containing IgA were nd concentrated to a vol of 2 to 3 ultrafiltration through an Amicon ne. (Amicon Corp., Lexington, luman IgG was purified from 200 ooled human serum, and hamster n 300 ml of normal hamster serum, e precipitations with 1/3 saturated um sulfate, passage through a ellulose column, and concentration con membrane filtration. Human s purified from 600 ml of pooled serum by the method of Cambier ler (9). The concentrated IgM was ographed twice on Sephadex G-200 and subjected to Amicon mem- ltration. The purity of each human globulin preparation was confirmed unodiffusion against antiserum to uman serum and against heavy ecific antisera to human IgA, IgG , and the protein concentration was ned by the biuret method. Immuno- nts were prepared with the purified globulins by the method of Avra- d Ternynck (10) using glutaralde- a cross-linking agent.

ractions of goat antisera to human G and IgM and hamster IgG were o the homologous immunoadsorb- d incubated at 37° for 30 min fol- y overnight incubation at 4° with shaking. After centrifugation at pm for 15 min, the supernatant ere removed, and the pellets were five times with 200 ml of PBS. The IgG was then eluted with 0.1 *M* HCl, pH 2.8. Eluted IgG was clari- 3000 rpm for 15 min, the pH was to 7.0, and the protein concentra- determined by the biuret method preparation had been held at 4° t.

The purified class-specific immunoglobulins were labeled with ^{125}I by the chloramine T method (11, 12). Complete details of radiolabeling have been published previously (1, 2). For use in the indirect RIA the reagents were diluted in PBS containing 5% normal goat serum to give 40,000 counts/min (CPM) in a volume of 0.1 ml.

RIA of viral antigen and antibodies. Details of the indirect solid phase RIA for detection of viral antigens in infected cells and brain specimens, and for assay of viral antibodies, are given elsewhere (1, 3, 4).

FA staining. The indirect FA staining method used in this laboratory has been described in detail (13).

Elution of antibody from brain tissue. A sample of brain tissue 3 to 4 mm in diameter was triturated with a glass rod in a polycarbonate centrifuge tube. Five milliter of PBS, pH 7.2, was added and the contents of the tube were stirred vigorously on a Vortex mixer, followed by shaking overnight at 4° on a wrist action shaker. After centrifugation at 35,000 rpm for 2 hr at 4°, the supernatant wash fluid was removed and saved for antibody assay. Fresh PBS was added to the tissue, and the above procedure was repeated for a total of five times, or until the wash fluid showed little or no antibody by RIA. Antibody was eluted from the washed brain tissue using either 2.5 *M* KI in PBS (5) or 0.1 *M* glycine-HCl, pH 2.8. Five milliliter of the eluting reagent was added to the tissue, and after thorough mixing and incubation at room temperature for 30 min, the mixture was centrifuged at 35,000 rpm for 2 h at 4° and the supernatant fluid was removed and diluted in PBS containing 1:5000 fetal bovine serum for antibody assay.

Before applying the elution methods to dissociate measles antibody from SSPE brain tissue, their reliability was confirmed using measles-infected HFDL cells and measles-infected hamster brain tissue to which specific antibody was absorbed. To 5 mm square samples of brain tissue or 0.5 ml of infected cell pack was added 0.05 ml of measles immune hamster serum followed, after thorough mixing, by 5 ml of PBS. After incubation for 1 h in a 37° water bath followed by shaking overnight

at 4° the mixture was centrifuged at 35,000 rpm for 2 h at 4°, the supernatant fluid was removed, and the tissue was washed in PBS and eluted as described above. Uninfected HFDL cells and hamster brain tissue were treated in parallel.

Results. Detection of measles virus antigen in brain tissue by RIA. Table I illustrates the specificity and sensitivity of the indirect RIA method for detecting measles virus antigen both in brain tissue of acutely infected hamsters and in the brain of an SSPE patient with a chronic measles virus infection. Controls consisted of brain tissue from uninfected hamsters and a human brain on which attempts to isolate virus or demonstrate viral antigen by FA were negative. In tests with measles-infected tissues the binding ratios obtained by dividing the CPM obtained with measles immune serum by those obtained with normal hamster serum were much above the 2.1 level considered as positive, and in tests with measles-negative brain tissues binding ratios were well below this level.

Of five coverslip preparations examined, each containing five sections of SSPE tissue, all were clearly positive by However, only 16 of 25 individual ad sections were positive by FA. These r are not directly comparable, since po five sections were examined by RIA individual sections by FA, but they dc cate that the method employed for gave positive results with all prepara while FA examination of individual se gave negative results in some instance

Elution of measles virus antibody l to infected cells and tissues. Table II results of preliminary studies design determine whether measles virus ant adsorbed onto infected cells or brain could be effectively eluted and then as by RIA. Infected HFDL cells appa contained greater amounts of acce measles virus antigen than did infected ster brain tissue, since greater amou antibody were adsorbed, and even eluted. Although some antibody was specifically adsorbed to the uninfecte and brain tissue, this was removed by ing, and none was recovered by el With infected cells and tissue, the five fluids showed successively less antibod large amounts of specific measles viru body were released from the washed by elution, and KI was more effecti this purpose than was acid elution.

Efforts were then made to elute antibody from the same human SSPE in which measles virus antigen was de strable by RIA, and also from a h brain from a fatal case of herpes si encephalitis. Table III shows in both the specificity of the eluted antibody f infecting virus. Again, KI was more tive than low pH for antibody elution. results indicate that RIA is applical

TABLE I. DETECTION OF MEASLES VIRUS ANTIGEN IN BRAIN TISSUE BY RIA.

Tissue examined	cpm with intermediate hamster serum (1:4000)		
	Measles virus immune hamster	Normal hamster	Binding ratio[a]
Normal hamster brain	400	339	1.2
Measles-infected hamster brain	3,064	347	8.7
Negative human brain	560	492	1.1
SSPE human brain	1,862	521	3.5

[a] cpm immune serum/CPM normal serum.

TABLE II. ABSORPTION AND ELUTION OF MEASLES VIRUS ANTIBODY.

Material treated with measles virus immune hamster serum	RIA titer to measles virus							
	Supernatant fluid after absorption	Wash #1	Wash #2	Wash #3	Wash #4	Wash #5	2.5 *M* KI eluate	0.1 cin el
Uninfected HFDL cells	400,000	50,000	10,000	2,500	<250	<250	<250	
Measles-infected HFDL cells	10,000	5,000	5,000	5,000	2,000	<250	>200,000	10
Uninfected hamster brain tissue	400,000	100,000	20,000	20,000	2,500	<250	<250	
Measles-infected hamster brain tissue	400,000	100,000	10,000	5,000	5,000	500	100,000	1

TABLE III. VIRAL SPECIFICITY OF IMMUNOGLOBULINS ELUTED FROM INFECTED HUMAN BRAIN TISSUE.

Infecting virus	Test virus[a]	RIA titer to test virus: Wash #1	Wash #2	Wash #3	Wash #4	Wash #5	2.5 M KI eluate	0.1 *M* glycine-HCl eluate
Measles[b]	Measles	2,000,000	200,000	100,000	20,000	2,000	100,000	40,000
	HSV	<250	<250	<250	<250	<250	<250	<250
HSV	Measles	2,500	<250	<250	<250	<250	<250	<250
	HSV	25,000	500	500	250	250	25,000	2,500

[a] Infected HFDL cells.
[b] SSPE patient.

the demonstration of both measles virus antigen in the same brain tissue.

Studies on the classes of immunoglobulins present in brain eluates. The first wash fluid and KI and glycine-HCl eluates from the SSPE brain tissue were applied to measles virus-infected cells, and the radiolabeled immune globulins specific for human IgA, IgG and IgM were used to detect antigen-antibody reactions. With each preparation positive results were obtained only with the anti-IgG reagent. The ability of the reagents to detect measles antibody in all three classes of immunoglobulins was confirmed by parallel testing of an early serum from a measles virus infection.

To examine the possibility that high concentrations of IgG antibody in the eluates might be inhibiting reactivity of IgM or IgA antibody, wash fluids and eluates, dialyzed against PBS, were subjected to sucrose density gradient centrifugation, and the gradient fractions were assayed for measles virus antibody by RIA using the heavy chain-specific reagents. As shown in Table IV, only the anti-IgG reagent gave positive results with the fractions. Only IgG was demonstrable in the eluates and gradient fractions by immunodiffusion against antisera to human IgA, IgG and IgM.

As a control on the ability of the gradient to separate viral antibody of different classes, an early serum from a rubella infection was centrifuged in parallel with the wash fluids and eluates, and fractions were assayed for rubella antibody by solid phase RIA using the heavy chain-specific reagents. IgM rubella antibody peaked in fraction 3, IgA antibody in fraction 6, and IgG antibody in fractions 8 and 9.

TABLE IV. CLASS OF MEASLES-SPECIFIC IMMUNOGLOBULIN IN SSPE BRAIN.

Sucrose density gradient fraction[a]	Reactivity in RIA of first wash fluid, KI and glycine-HCl eluates with radiolabeled antibodies to: IgG	IgA	IgM
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	+[b]	0	0
6	+	0	0
7	++	0	0
8	+++	0	0
9	++++	0	0
10	+++	0	0
11	+	0	0
12	+	0	0

[a] 1 = most dense, 12 = least dense.
[b] + = antibody titer of 1:1000.
++ = antibody titer of 1:2000.
+++ = antibody titer of 1:5000.
++++ = antibody titer of ≥1:10,000.

Discussion. The present study has extended the use of RIA for detecting viral antigen directly in clinical materials. It has previously been shown that RIA can be used for detecting and typing herpes simplex virus (HSV) directly in human brain tissue (1), and for demonstrating rabies virus antigen in brain tissue (B. Forghani, unpublished). The method can also be used for clear-cut and specific demonstration of measles virus antigen in SSPE brain tissue. The sensitivity of RIA for this purpose was as good as, and perhaps better than, that of FA staining. Further, by using the highly sensitive RIA method for detection of viral antibody in eluates from brain tissue, it was possible to demonstrate measles virus antibody in the same SSPE brain in which antigen was demonstrable.

Assay of viral antibodies in brain eluates by RIA has several advantages, the most notable of which is marked sensitivity, as

evidenced by the high antibody titers obtained. This makes it unnecessary to concentrate eluates for antibody assays (14), and the eluates are diluted >1:100 for antibody assay, making preliminary adjustment of pH or ionic strength unnecessary. The use of highly dilute eluates for antibody detection avoids the problems of nonspecific hemagglutinating or inhibitory activity which has been reported in other studies (14).

The sharp specificity of the RIA for detecting viral antibodies in brain eluates was clearly illustrated by the absence of reactivity of eluates for uninfected brain tissue or from brain tissue infected with a different virus. It is of particular interest that the eluates from the SSPE brain which had high levels of measles-specific activity failed to react in RIA with cells infected with HSV. HSV-infected cells have been reported to produce IgG receptors (15) similar to those produced by cells infected with human cytomegalovirus (16) which bind nonantibody IgG. While IgG receptors on CMV-infected cells may cause some nonspecific binding of IgG in FA reactions (16) and in RIA reactions (3), we have seen no evidence of such nonspecific reactivity in RIA tests with HSV, either in previous studies (1, 2, 4) or the present one.

In this study KI was found to elute two to ten times more viral antibody from brain tissue than was dissociated by low pH. The combination of elution with chaotropic ions and a sensitive RIA for antibody assays may prove useful in studies aimed at identifying a viral etiology of certain chronic neurological infections through the demonstration of specific viral antibody in eluates from diseased tissues.

The highly purified radiolabeled antibodies specific for the various classes of human immunoglobulins demonstrated measles virus antibodies only of the IgG class in SSPE brain eluates. This is in accord with results obtained by Vandvik *et al.* (17) using somewhat different approaches to characterize measles virus antibodies in cerebrospinal fluid and brain extracts from SSPE and multiple sclerosis patients.

Summary. A solid phase radioimmunoassay (RIA) procedure was used to detect measles virus antigen in brain tissue of experimentally infected hamsters and in a human SSPE brain. RIA was also used to detect measles virus antibody in eluates from the same SSPE brain. The antibody in the eluates was sharply measles-specific, showing no reactivity by RIA with HSV-infected cells. Similarly, antibody eluted from a HSV-infected human brain reacted only with the infecting virus. KI eluted two to ten times as much viral antibody from infected brain tissues as was dissociated by low pH. Radiolabeled antibodies to human IgA, IgG and IgM which had been purified and concentrated by immunoadsorption with purified immunoglobulins were used in the RIA system to study the classes of immunoglobulins present in SSPE brain eluates; only measles antibody of the IgG class was demonstrable.

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ɜrentiation of Serum Antibodies to Herpesvirus Types 1 and 2 by Radioimmunoassay (40036)

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nplex virus types 1 (HSV-1) 2) share common antigens and specific antigens (1). Much of directed against HSV-2 also antigens shared with HSV-1 ɔn with either virus type. This ɔgic diagnosis of HSV-2 infec- because many adults have had tions before becoming infected (2). We were able to detect HSV-1 and HSV-2 using a solid nmunoassay (RIA). By adsorp- ɪiniature affinity columns of ystyrene coated with HSV-1, ıe antibody directed against ɜns could be removed, allowing ıntibody to HSV-2 type specific RIA.

and methods. Tissue culture. ɔnsisted of monkey kidney ls grown in basal medium Ea- % fetal calf serum (FCS), glu- *M*/ml) and neomycin (20 μg/ of HSV-1 (Patton) and HSV-2 indly provided by Dr. F. Rapp. yers in 32-ounce prescription infected with virus and incu- >90% of all cells showed cyto- (about 20 h). Infected cells twice with phosphate buffered and frozen at −70°.

s. Adsorbents were prepared polystyrene test tubes (Falcon, ıto a fine powder with the aid omnimixer. This powder was PBS and approximately 40 ml placed into a 100 ml beaker. m three 32-ounce bottles of ted cells was thawed, sonicated vith 20 ml PBS. This was mixed ıetic stirrer for 1 h at room The adsorbent was poured ɜieve (Cistron Corp., Elmsford, washed with 100 ml of 10% l adsorbent was prepared with 10% FCS in place of HSV-1. Adsorbent (approximately 4 ml) was then put into small disposable columns (Quik-Step columns, Isolab, Inc., Akron, OH) and were ready for immediate use. In experiments where adsorption was utilized, sera were diluted 1:200 in 10% FCS in PBS and tumbled overnight at 4° either with HSV-1 coated polystyrene (referred to as adsorbed sera) or with FCS coated polystyrene (referred to as unadsorbed sera).

Serum samples. Forty-eight coded serum samples from women with different histories of herpetic infection were obtained for analysis. The first group of sera was collected from eighteen nuns who denied ever having sexual intercourse. A second group was collected from eighteen prostitutes, all of whom admitted to having multiple sex partners. The third group of sera was collected from ten asymptomatic women from whom HSV-2 was isolated and identified during blind screening (3). Also included in these forty-eight sera were one serum from an individual negative for HSV-1 and HSV-2 and sera from two individuals infected with HSV-2 confirmed by virus isolation from genital lesions. It was reasoned that the nuns would be low risk for HSV-2 infections and the prostitutes would be at high risk for HSV-2 infections, although virus isolation data was not available on these two groups. Two uncoded sera were selected for preliminary experiments from two persons who had previous infections with HSV-1 only or HSV-2 only, confirmed by virus isolation.

RIA Procedures. All sera were diluted 1:200 in 10% FCS in PBS and tested with HSV-1, HSV-2, and VERO antigens as previously described (4). Briefly, plastic-coated beads were coated with antigen and reacted overnight with patients' sera. Excess serum was washed off and beads were reacted 4 h with ^{125}I-labeled antiglobulin, washed and the radioactivity on the beads

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counted in a α-counter. All washings were carried out with a specially designed magnetic transfer device capable of performing multiple assays simultaneously (5). The HSV-1 antigen used to coat beads was diluted to give a reaction in cpm approximately equal to the reaction with HSV-2 antigen when tested with pooled serum from patients infected with HSV-1 followed by HSV-2.

^{51}Cr release test. Antibodies to HSV-1 and HSV-2 infected cells were quantitated using the release of ^{51}Cr in the presence of guinea pig complement (6). To detect type specific antibodies to virus infected cells, the sera were adsorbed with cells infected with the heterologous virus type (6).

Results. In order to determine the effect of serum adsorption with HSV-1 coated polystyrene, two sera were tested. The first serum was obtained from a person infected with HSV-1 only. Before adsorption this serum gave a reaction of about 2000 cpm higher with HSV-1 antigen than with HSV-2 antigen (Fig. 1). Essentially all antibody to HSV-1 type specific and common antigens were removed by adsorption as indicated by no reaction with HSV-1 or HSV-2 antigens following adsorption. A second serum from an individual infected with HSV-2 only was also tested. This serum reacted approximately 4000 cpm higher with HSV-2 antigen than with HSV-1 antigen before adsorption, an opposite pattern than that

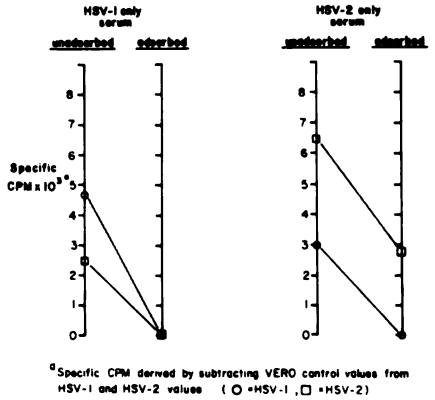


FIG. 1. Effect of serum adsorption with HSV-1 coated polystyrene. Specific cpm were derived by subtracting VERO control values from HSV-1 and HSV-2 values, ○——○ = HSV-1, □——□ = HSV-2.

observed with the serum from the indiv infected with HSV-1 only. Followin sorption, essentially all antibody to the mon antigen of HSV-1 was remov indicated by lack of detectable reactior HSV-1 antigen. Antibody to HSV-2 ar was then clearly detectable at approxin 2700 cpm above background.

Serum samples from forty-eight w were then tested under code for ant to HSV-1, HSV-2 and uninfected V control cell antigens. These sera wer tially tested without adsorption by Serum reactivities in cpm with each ar were determined and ratios were calcu by dividing cpm obtained with HSV-2 gen by cpm obtained with HSV-1 ar (Table I). The HSV-2/HSV-1 ratios r from −1.0 to +4.6, those sera with ratios giving higher reactions with H than with HSV-2 antigen. As the H HSV-1 ratio becomes larger, serum re ity with HSV-2 antigen increases relat the reaction with HSV-1 antigen.

A plot of this RIA data indicates at two populations within the distributi HSV-2/HSV-1 ratios (Fig. 2). Those with low ratios indicate a preponderar antibody to HSV-1 antigen and those higher ratios indicate more antibo HSV-2 antigen. An intermediate gro sera between the two populations was t by serum adsorption in an attempt t move antibody to common antigens identify those women with antibo HSV-2 type specific antigens.

The data in Table II show the H HSV-1 ratios before and after adso and the origin of the serum samples the code was broken. If a serum g ratio of a greater than or equal to 1 was considered positive for antibo HSV-2 type specific antigen. The r for using this criterion is that the H and HSV-2 antigens used in this RIA diluted to give approximately equal tions when tested with pooled sera individuals infected with HSV-1 fol by HSV-2. Six of the sera tested by a tion converted to ratios of greater tha after adsorption; demonstrating the of adsorption, which was to remove body directed against the shared an

LE I. RADIOIMMUNOASSAY OF CODED SERUM SAMPLES

cpm × 10^3			
:r HSV-1 antigen	HSV-2 antigen	Cell control antigen	Ratio[a] HSV-2/HSV-1
1.0	0.4	0.7	−1.0
1.0	0.6	0.4	0.3
2.7	1.2	0.4	0.4
2.4	1.1	0.5	0.4
4.0	1.9	0.5	0.4
1.9	1.1	0.5	0.4
4.6	2.4	0.7	0.4
3.0	1.7	0.6	0.5
3.2	1.8	0.6	0.5
3.5	1.9	0.5	0.5
4.1	2.2	0.5	0.5
2.8	1.7	0.6	0.5
4.3	2.4	0.5	0.5
5.2	3.1	0.7	0.5
3.6	2.2	0.6	0.5
4.1	2.3	0.6	0.6
4.2	2.6	0.6	0.6
4.4	2.8	0.6	0.6
3.7	2.4	0.6	0.6
5.6	3.6	0.5	0.6
5.0	3.4	0.9	0.6
4.9	3.3	0.7	0.6
2.5	1.7	0.4	0.6
5.2	3.5	0.7	0.6
3.7	2.5	0.4	0.6
4.5	3.1	0.6	0.6
3.5	2.3	0.5	0.6
3.0	2.2	0.4	0.7
4.2	3.1	0.6	0.7
6.2	4.6	0.9	0.7
3.3	2.7	1.0	0.7
5.6	4.6	1.1	0.8
2.8	2.3	0.5	0.8
6.2	5.1	0.5	0.8
5.6	4.6	0.3	0.8
4.3	3.7	0.6	0.8
3.5	3.0	0.4	0.8
4.9	4.3	0.7	0.9
2.8	2.5	0.5	0.9
7.4	6.7	0.3	0.9
4.7	4.8	0.8	1.0
2.7	2.9	1.0	1.1
5.1	7.3	0.5	1.5
1.6	2.1	0.8	1.6
1.5	2.3	0.7	2.0
2.8	4.6	1.0	2.0
2.8	5.3	0.6	2.1
1.4	3.9	0.7	4.6

:ans HSV-2 counts minus VERO counts divided
'-1 counts minus cell control counts.

t HSV-2 type specific antibody could
ected.

:r breaking the serum code it was
ed that serum numbers 4–12 came from asymptomatic women from whom HSV-2 was isolated during blind screening. The second group of sera, numbers 13–30, came from nuns and the third group of sera, numbers 31–48, came from prostitutes. Also included were one seronegative control (serum number 1) and two seropositive controls (sera numbers 2 and 3) for HSV-2 confirmed by virus isolation and serum neutralization. It was reasoned that the nuns would be at low risk for HSV-2 infections and the prostitutes would be at high risk for HSV-2 infections, although virus isolation data were not available on these two groups.

The results summarized in Table III indicate which serum samples were positive for HSV-2 antibody measured by RIA and ^{51}Cr release methods. All sera (except #32) were titered, then diluted to contain 5 units of antibody, adsorbed with HSV-1 infected cells and then assayed in the ^{51}Cr release test against HSV-2 infected cells. Greater than 2% specific ^{51}Cr release is considered significant (6) for the presence of HSV-2 antibody. It can be concluded that all the nuns' sera were negative for HSV-2 antibody by both assays, meaning that there were no false positive. Ten of eighteen prostitutes' sera were positive by RIA compared with three positives out of seventeen prostitutes' sera tested by ^{51}Cr release. Of the nine asymptomatic women, three were positive by RIA and two were positive by ^{51}Cr release. This group of asymptomatic women would contain those individuals likely to make little if any detectable antibody response to an inapparent HSV-2 infection for reasons in the discussion.

A comparison of RIA and ^{51}Cr release assay was also made by plotting HSV-2/HSV-1 RIA ratios versus II/I cytotoxic antibody indices obtained by ^{51}Cr release assay (Fig. 3). The nuns' sera gave lower ratios and indices than did the prostitutes and asymptomatic women. A partial correlation between the two methods appears to exist except for four prostitutes' sera and one serum sample from an asymptomatic woman, all of which had high HSV-2/HSV-1 RIA ratios.

Discussion. It has been reported that persons infected with HSV-1 or HSV-2 alone

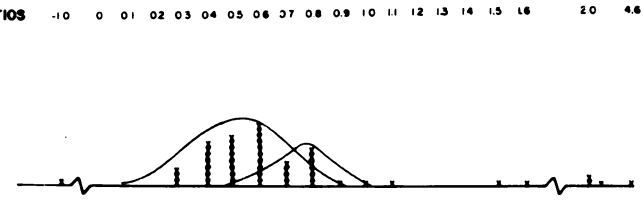


FIG. 2. RIA ratios, HSV-2/HSV-1, on coded serum samples. Each X represents a serum sample. RI were calculated according to the formula: HSV-2 minus VERO ÷ HSV-1 minus VERO. Values (in cp determined by RIA with each of the forty-eight sera using HSV-1, HSV-2 and VERO antigens.

TABLE II. EFFECT OF SERUM ADSORPTION WITH HSV-1 COATED POLYSTYRENE. RATIO MEANS HSV-2 CPM DIVIDED BY HSV-1 CPM.

Unadsorbed			Absorbed		
Serum No.	Group	Ratio	Serum No.	Group	Ratio
47	prostitute	0.6	10	asymptomatic	0.6
44	prostitute	0.7	44	prostitute	0.6
18	nun	0.7	6	asymptomatic	0.7
42	prostitute	0.7	22	nun	0.8
38	prostitute	0.7	42	prostitute	0.8
46	prostitute	0.7	18	nun	0.8
33	prostitute	0.8	47	prostitute	0.8
40	prostitute	0.8	9	asymptomatic	0.8
11	asymptomatic	0.8	33	prostitute	0.9
9	asymptomatic	0.8	43	prostitute	1.1
43	prostitute	0.8	8	asymptomatic	1.1
8	asymptomatic	0.8	46	prostitute	1.1
22	nun	0.9	40	prostitute	1.2
6	asymptomatic	0.9	11	asymptomatic	1.2
10	asymptomatic	0.9	32	prostitute	1.3
31	prostitute	1.0	38	prostitute	1.5
32	prostitute	1.1	31	prostitute	2.0

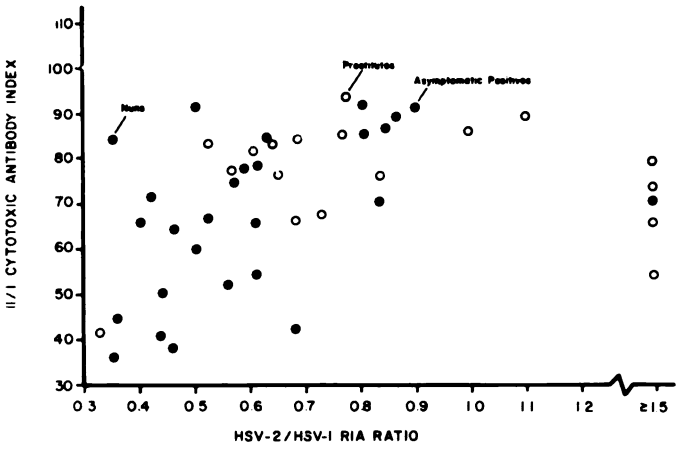
produce a population of antibody of which approximately 80% is directed against the cross-reacting or common antigen shared by the two viruses. The remainder of the antibody (20%) is produced against type specific antigens (6). Most humans become exposed to HSV-1 before reaching sexual maturity, at which time a relatively smaller percent of the population acquire infection with HSV-2. If a person has antibody to HSV-1 and becomes infected with HSV-2, the antibody production shifts to 90% against shared antigen indicating amestic response, 5–10% against specific antigens and a minimal resp HSV-2 specific antigens as measu complement dependent lysis of i cells. A possible explanation for asymptomatic women positive by virus isolation and negative by RIA be that high levels of preexisting neutralizing antibody to the commc gen obtained from an earlier infecti HSV-1 prohibits these women fr sponding to the HSV-2 type specif gens upon infection with this virus.

In conclusion, the advantages of tl for serological detection of past HS fection include its simplicity and app ity to testing a large number of seru ples rapidly at low cost without the purified antigen. By using HSV-1 polystyrene adsorbents in miniature columns, antibody to the shared anti HSV-1 and HSV-2 could be re These procedures enhance detectior tibody to HSV-2 type specific antig lowing a better assessment of the h response to infection with HSV-1 an 2. Finally, this technique is an ec

TABLE III. HSV-2 ANTIBODY IN CODED SAMPLES.

		HSV-2 Antib	
Serum No.	Source	Ria Positive/Total	^{51}Cr Po T
4–12	Asymptomatics with HSV-2 Isolation	3/9	
13–30	Nuns	0/18	
31–48	Prostitutes	10/18	

[a] Sera diluted to contain 5 units of antib sorbed with HSV-1 infected cells and then the ^{51}Cr release assay (6).



. Comparison of RIA and ^{51}Cr release assay. HSV-2/HSV-1 RIA ratios were determined by dividing activity in cpm with HSV-2 antigen by serum reactivity with HSV-1 antigen. II/I cytotoxic antibody ere calculated accordingly = II/I index = titer (HSV-2) $\log_{10}$/titer (HSV-1) $\log_{10} \times 100$. Titers were ed by ^{51}Cr release (6).

ive to the use of a highly purified ecific antigen recently reported by *al.* (7).

nary. Antibody against herpes virus (HSV-1) and 2 (HSV-2) was de- n a coded test of forty-eight coded sera by radioimmunoassay (RIA). ly to antigens shared between the is types could be removed by adsorp- h HSV-1 coated polystyrene allow- ection of HSV-2 specific antibody. positives were observed in eighteen at low risk for HSV-2 infection. specific antibody was detected in eighteen women who were at high HSV-2 infection. Antibody to HSV- etected in three of ten asymptomatic Biological variation in response to HSV-2 could provide an explanation for those asymptomatic women who were positive by virus isolation, but negative for HSV-2 type specific antibody by RIA.

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Influence of Dietary Fat and Carbohydrate on Food Intake, Body Weight and Bo Fat of Adult Dogs[1] (40037)

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Certain strains of rodents become fatter when fed high-fat rather than high-carbohydrate diets while other strains are able to consume a high-fat diet without an appreciable increase in body fat content (1-4). The obesity prone rodents are less able to regulate their food intake when fed a high-fat diet; additionally, consumption of a high-fat diet has also been shown to increase the efficiency of energy utilization in pair-fed rodents (5, 6). Less attention has focused on the interrelationship of the dietary fat to carbohydrate ratio and the development of obesity in other species.

The influence of the dietary fat to carbohydrate ratio on body weight and body fat has been studied in young growing dogs (7). The fat-free body weight gain was not influenced, but accumulation of body fat tended to be elevated in dogs fed high-fat diets. Body fat averaged 29% of body weight in the young dogs fed diets with more than 50% of energy from fat while the body fat content averaged 23% in dogs fed the diets which contained less than 50% of energy from fat. The dogs were only 10-months-old at the end of the 8-month study; had the diets been fed to adult dogs, body fat content might have been altered to a greater extent.

The purpose of the present study was to examine the influence of a high-fat diet (51% of energy from fat) and a high-carbohydrate diet (59% of energy from carbohydrate) on food intake and on body weight and body fat changes in adult dogs. Dogs were fed these diets for 25 weeks.

Materials and methods. Three-year old female beagle dogs[2] were housed in individual cages with raised floors. Lights in temperature-controlled (22-23°) room on from 7 AM to 7 PM and off from 7 P 7 AM. Prior to the present study the were fed approximately 750 kcal daily 110 days. The composition of this nutri ally adequate (8) canned diet has described previously (Table I, Diet Ref. 7). The dogs approximately m tained their weight during this 110-day riod. Food and water were offered *ad* during the present study. Dogs were once daily. Body weights were reco weekly.

Two semipurified diets were prepa one diet was high-fat and the other l carbohydrate. Each diet was formulate contain 20% of energy from protein. proximately 50% of the protein in the were derived from isolated soybean pr and 50% from beef kidneys. Corn oil of energy) was added to provide a sour essential fatty acids. Corn starch and ta were added to provide the desired carb drate to fat ratio. A mineral and vit supplement[3] and cellulose (6% of dry ter) were added to each diet. The were canned.

At monthly intervals samples of each were obtained and analyzed for mois by drying to a constant weight at 50° gross energy with a bomb calorimeter for nitrogen by the Kjeldahl proced Protein was determined by multiplying

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[2] Laboratory Research Enterprises, Inc., Kalamazoo, MI.

[3] Vitamin premix was added at the level of 55 kg diet. The vitamin premix contained per g: vi A-5344 IU; vitamin D_3 = 132 IU; vitamin E-2 vitamin B_{12}-5.8 mg; thiamin-741 mg; riboflavin mg; pyridoxine-88 mg and choline chloride-253 mineral premix was added at the level of 465 m kg diet. The mineral premix contained in mg potassium chloride-933; magnesium oxide-43; c sulfate-10; cobalt sulfate-9; potassium iodate- manganese sulfate-2. Additionally, dicalcium phate, iodized salt and potassium chloride were at the levels of 1.4, 1.0 and 0.36% of the diet.

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LE I. COMPOSITION AND DIGESTIBILITY OF DIETS FED TO ADULT DOGS.

	Diet	
	1	2
er, g/g diet	25.4	25.4
ıergy, kcal/g	1.43	1.16
digestibility,	85.7 ± 0.4[a]	89.5 ± 0.6[b]
zable en- , %[c]		
ı	20	18
	51	23
hydrate	29	59

ın ± SEM for 7 dogs.
ificantly different from values obtained when
e fed diet 1 ($P < 0.05$).
Materials and Methods for method of calcula-

n content of the diet by 6.25. The
age of metabolizable energy from
, fat and carbohydrate in each diet
ıen determined as previously de-
(7).
body water content of each dog was
ined at the start and again at the end
tudy. Each dog was fasted overnight
ɔn injected intravenously with 2 ml
e containing 50 μCi ^3H_2O. A blood
was obtained 3 h later and the
activity of the plasma was deter-
The body water content and fat-free
ıass were then calculated as previ-
escribed (7). Body fat content was
ed by subtracting the fat-free mass
e body weight of the dog.
apparent digestible energy value of
et was determined during the 20th
f the study. Total fecal collections
ach dog were continued for 5 days.
vere dried at 50°. A bomb calorime-
then utilized to determine total fecal
The difference between the gross
intake and the fecal energy output
the 5 day period represented the
ıt energy digestibility of the diets.
data were treated statistically by the
's *t* test.

lts and Discussion. The composition
ıergy digestibility of the diets are
ed in Table I. Both diets contained
ry matter. The gross energy content
1, the high-fat diet, was 23% higher
iet 2, the high-carbohydrate diet. Digestibility of energy in the high-carbohydrate diet was 4% greater than observed when the high-fat diet was fed. This difference in digestibility was unexpected. In a previous study with growing dogs a high-carbohydrate diet of similar composition to diet 2 exhibited slightly lower digestibility than did diets higher in fat (7). Differences in energy intake may influence digestible energy values; however, dogs in both groups consumed similar amounts of food during the digestion trial. Gross energy intake of diet 1 and 2 averaged 4185 ± 271 and 4506 ± 157 kcal, respectively, for the 5-day period. Based on the analyzed composition, diet 1 was slightly higher in protein than was diet 2. Dogs fed diet 2 consumed an adequate quantity of protein (40 g per day), thus it is unlikely that the small difference in protein content of the diets influenced the results.

The dogs had been fed a restricted amount of food prior to this study; consequently, food intake was high during the first several weeks of the study (Fig. 1). Dogs fed the high-fat diet consumed more energy than did dogs fed the high-carbohydrate diet during this period; however, the differences were not significant. After approximately 6 weeks, energy intake stabilized and remained rather constant during the remainder of the study. The average daily energy intake of the dogs during the first 12 weeks and during the last 13 weeks of the study is presented in Table II. None of the differences in energy intake were statistically significant.

Body weight changes of the dogs are presented in Fig. 1. Both groups of dogs increased their body weights during the study; but by 10 weeks the increase in body weight of dogs fed the high-fat diet was significantly greater than observed when the high-carbohydrate diet was fed. The increase in body weight of dogs fed the high-fat diet during the first 12 weeks was twice the increase observed when the high-carbohydrate diet was fed (Table II). During the last half of the study the rate of body weight gain decreased in both groups of dogs, but dogs fed the high-fat diet still gained more than did dogs fed the high-carbohydrate diet. This greater body weight gain in dogs fed the high-fat diet occurred even though

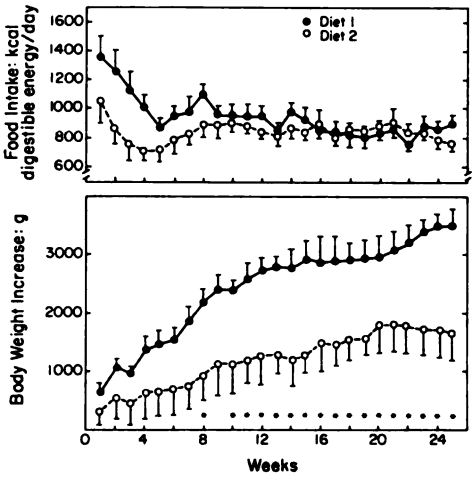

FIG. 1. Influence of diet composition on daily food intake and body weight changes of adult dogs. Each point represents the mean ± SEM for seven dogs. * indicates significant differences ($P < 0.05$).

TABLE II. INFLUENCE OF DIET COMPOSITION ON FOOD INTAKE, BODY WEIGHT AND BODY FAT OF ADULT DOGS.[a]

Parameter	Diet 1	Diet 2
Initial body wt, kg	10.26 ± 0.43	10.22 ± 0.33
Initial body fat, kg	2.98 ± 0.50	3.10 ± 0.36
Food intake, kcal digestible energy/day		
Weeks 0–12	1038 ± 59	841 ± 66
Weeks 13–25	855 ± 42	832 ± 41
Body weight gain, kg		
Weeks 0–12	2.75 ± 0.24	1.25 ± 0.45[b]
Weeks 13–25	0.74 ± 0.12	0.36 ± 0.08[b]
Body fat gain, kg	2.78 ± 0.44	1.26 ± 0.41[b]

[a] Results expressed as Mean ± SEM for seven dogs.

[b] Significantly different from values obtained when dogs were fed diet 1 ($P < 0.05$).

energy intake of the two groups of dogs during the last half of the study was virtually identical (Table II). Dogs fed the high-fat diet consumed less than 3% more energy than did dogs fed the high-carbohydrate diet during this period. Body fat gain averaged 78–80% of body weight gain (Table II). This observation indicates that the increase in body weight observed in these adult dogs could be accounted for by gain in adipose tissue as adipose tissue from mature animals contains approximately 85% fat (9).

Dogs fed the high-fat diet consumed only 13% more energy during the study, but gained 117% more energy (assumed that the body weight gain was adipose tissue and that the adipose tissue contained 7700 kcal/kg). Dogs fed the high-fat diet retained approximately 16% of the digestible energy consumed, whereas dogs fed the high-carbohydrate diet retained only 8% of the digestible energy consumed. These results agree with the suggestion that the energy cost of fat deposition is greater when high-carbohydrate diets are fed than when high-fat diets are fed (5, 6, 10). However, as discussed in the next paragraph, an alternative explanation of the results is also plausible.

The metabolizable energy requirement of these adult, relatively inactive, dogs was estimated to average 840 kcal per dog per day (8). This value closely approximates the actual energy intake of the dogs (Table II). Consequently, even when the dogs were in positive energy balance, only a small fraction of the energy consumed by the dogs would be available for body fat gain. This small increment in daily energy intake, which might be practically nonmeasurable, could if allowed to accumulate for months, result in significant increases in body fat. The difference in energy intake between dogs fed diet 1 and diet 2 during the first 12 weeks averaged 197 kcal per day; during the last 13 weeks the difference was 23 kcal per day. If it is assumed that this additional energy consumed by the dogs fed the high-fat diet was deposited as adipose tissue (assume 7700 kcal/kg adipose tissue), then it would be possible to explain the entire difference in weight gain of the dogs on the basis of the small nonstatistically significant difference in food intake. The increased energy intake of the dogs fed the high-fat diet, if deposited as adipose tissue, would account for 2.42 kg adipose tissue gain. The observed difference in gain was 1.88 kg, suggesting that the increased energy intake in dogs fed the high-fat diet was utilized for adipose tissue gain with an efficiency of 78%.

It would be necessary to pair-feed the

to determine whether the increased
eposition observed in the dogs fed the
-fat diet resulted from an increased ef-
ncy of energy utilization or from the
l difference in energy intake. Possibly
factors contributed to the results ob-
d in the present study.

mmary. Adult female dogs were fed
ibitum for 25 weeks a high-fat diet
% of energy from fat) or a high-carbo-
ate diet (59% of energy from carbohy-
e). Dogs fed the high-fat diet gained
e body weight than did dogs fed the
-carbohydrate diet. In both groups of
78–80% of the increase in body weight
fat. The high-fat diet may have been
ed more efficiently for body fat gain
the high-carbohydrate diet; alterna-
y, it is possible to explain the increased
fat accumulation in dogs fed the high-
liet on the basis of the small observed
rence in energy intake. Dogs fed the
-fat diet consumed slightly more energy
%) which resulted in the accumulation
ore than twice the amount of fat accu-
ted in dogs fed the high-carbohydrate
during the 25 week study.

The authors wish to acknowledge the technical assistance of Kathleen Muiruri.

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Distribution Kinetics of Urea Between Plasma and Red Blood Cells of Normal Human Blood[1] (40038)

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Urea passes through all the membranes of mammalian organs without any apparent resistance and the $t_{\frac{1}{2}}$ for the entry of urea into the human red blood cells (RBC) from an isosmolar solution is 0.053 sec at 21° (1). In spite of this rapid transfer of urea a substantial variation between plasma urea and cell urea concentrations under physiological conditions had been reported (2-5). This concentration difference was postulated as due to the existence of urea in various states. However no attempts were made to distinguish between the freely exchangeable urea and any relatively bound urea. Model dialysis experiments (6, 7) suggest that the clearance rate of urea from plasma may not be the same as that from RBC. Using isotopic techniques we have studied the *in vitro* kinetics of urea distribution between these two blood compartments and the *in vivo* fractional turnover rates of labeled urea. There is no concentration difference in the exchangeable (free) urea content between the cell water and plasma water. The difference found between cell urea and plasma urea sp act in all our [^{15}N] experiments suggest the existence of more bound urea in cells than in plasma.

Materials and methods. (a) *In vivo study.* Healthy normal volunteers, with their written consent, were admitted to the Surgical Metabolism Unit of Columbia-Presbyterian Medical Center and placed on a metabolic balance program for two days. They were provided with regular diet containing enough nitrogen to maintain balance. On the third day, 100 mg of pyrogen free urea (98 atom % [^{15}N]) in 15 ml normal saline was injected intravenously. Just prior to the injection a control sample of venous blood was taken. After dose injection blood samples (10-15 ml) were withdrawn in heparinized syringes at 10, 30, 60, 90, 120, 180, 210, 300, 360, 420, 480, 540, and 660 min and immediately centrifuged to separate the plasma from the cells. In one subject (MS), two days after this study of the distribution of the injected [^{15}N]urea between RBC and plasma (MS-1), when the level of the labeled urea attained almost the base value, [^{15}N]-L-alanine was injected and the sp act of the endogenously produced [^{15}N] urea in RBC and plasma were determined (MS-2).

The RBC and plasma fractions were diluted with deionized water and deproteinized by the Somogyi procedure using $Ba(OH)_2$ and $ZnSO_4$. After centrifugation, the supernatant fractions were shaken for 15 min with 2 ml of permutite to remove the ammonia of the blood and then treated in duplicate with urease in a Conway micro diffusion system. One milliliter of saturated K_2CO_3 was added to liberate ammonia which was then trapped on a filter paper strip (7.0 × 0.3 cm) moistened with a few drops of 1 *N* H_2SO_4. Sufficient time (about 8 hr) was allowed for the diffusion of ammonia. The paper strip was then removed and treated with sodium hypobromite in a micro Rittenberg gas tube. The liberated nitrogen gas was analyzed for its isotopic content in a 60° mass spectrometer which is capable of giving a precision of about five parts per 100,000. This procedure of trapping ammonia in a filter paper and using a micro Rittenberg tube minimizes any air contamination and provides maximum response for the peak heights of masses 28, 29 and 32. The replicate measurements

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agree within 0.5%. Reference nitro- (0.365±0.002 [^{15}N]atom %) was d at regular intervals to check the of the instrument. Atoms % excess etermined by subtracting values for [^{15}N] in control samples taken prior injection. In some samples of JL-1 -2, the [^{15}N] atom % excess in the blood was also measured to check ope distribution by material balance.

n vitro study. Twenty milliliter of drawn heparinized normal venous vas kept in a water bath at 37±0.2° red slowly with a magnetic stirrer. 1 to 2 mg of [^{14}C] urea (sp act 0.27 ;) was dissolved in 0.1 ml saline and y mixed with the blood. The osmo- hange due to the addition of this olume of tracer solution should be icant and any change in the hemato- y be considered negligible. Using bumin as a marker we have found most complete separation of the and cells was possible by spinning y hematocrit tubes (75 mm × 1.5 .) filled with blood in a micro centri- nternational Micro-Capillary Centri- odel MB). Samples were withdrawn , 15, 45, 60, 90, 120 and 180 min s in hematocrit tubes of uniform e end of which were then sealed and r three min. After cutting with a file arding the interface, known lengths atocrit tubes containing plasma and gments were collected separately. ere then crushed and deproteinized ng with 2 ml deionized water, 1 ml $Ba(OH)_2$ and 1 ml 0.3 *N* $ZnSO_4$ yi procedure). After centrifugation, r supernatant was filtered and a 0.5 licate of the filtrate was mixed with Bray's solution and counted in a cintillation counter (Model Mark II, Chicago Corp., Des Plaines, Ill). nples were counted for at least 10^4 Internal standards were used for cy calculations. The recovery of ea during the Somogyi precipitation cells or plasma and any dragging of rea by the proteins was checked dently by deproteinizing the cells or each containing equal amounts of ea and then counting as described before. No differences were found between the control (583±10 dpm) and the plasma (579±15 dpm) or cell samples (590±17 dpm), confirming the same recovery from deproteinizing cells and plasma as well as the absence of any drag of [^{14}C] of urea by the proteins.

Similar *in vitro* experiments were done with [^{15}N] urea, and the sp act (^{15}N atom % excess) in the separated cells and plasma were determined as described before in the *in vivo* study.

We have also measured the efflux of the labeled urea from the cells into the surrounding medium of plasma. A 25 ml portion of the fresh blood (PUN = 11.0 mg%, Hct = 30.1) was centrifuged for 20 min and the plasma was separated from the cells. One portion of the plasma was used to reconstitute the blood and the rest was analyzed for its water content. Another 25 ml original blood was equilibrated at 37° for 3 hr with about 1 mg of [^{14}C] urea dissolved in 0.1 ml normal saline. The labeled cells were then separated by centrifugation and mixed with sufficient original nonradioactive plasma to reconstitute blood. The efflux of [^{14}C] urea from the cells of this reconstituted blood was then determined as described before for the influx experiments.

The water content of the cells and plasma in all the samples was measured in duplicate by drying the diluted samples under vacuum at 70° to constant weight. The specific gravities of blood and plasma were also measured. The volume percent of water in cells (range 69.5–71.3) and plasma (range 92.5–93.7) corresponds to a water distribution ratio of 1.25–1.30 between equal volumes of plasma and cells with a mean (±SEM) value of 1.288±0.007.

Results. The time course of the disappearance of the injected urea from plasma and cells for a typical subject (BW) is illustrated in Fig. 1. The sp act of the plasma urea was consistently higher than that of the cell urea. Both the plasma and cell urea curves followed a two exponential decay. The slopes of the plasma and cell curves on a semilog scale are not parallel to each other but tend to converge during the initial period of about 3 hr and then diverge for all

the subjects studied. These differences are significant ($P < 0.01$) when examined by paired t tests. By least squares method the logarithm of sp act was regressed with time and the calculated slopes which are the fractional turnover rates of urea from plasma or cells are summarized in Table I. The sp act ratio between plasma and cells (P/C) was always found to be greater than one. There was a rather slow change in the P/C ratio during the study (Table I) and its range was about ±4% of the mean. The whole blood urea sp act measured in some of the samples from JL-1 and JL-2 were found to be lower than that of corresponding plasmas but higher than that of cells and the relative sp act in blood, plasma and RBC was 1.00:1.06:0.92.

In the *in vitro* experiments the addition of a tracer will have negligible effect on the membrane hydraulic conductivity or on the solvent flow through the membrane (8). Under these experimental conditions, the predominating factor is the diffusional flow of urea. Figure 2 shows the distribution ratio (Mean±SEM) of the radioactive [^{14}C] between equal volumes of plasma and cells as a function of time in influx (n=4) and efflux (n=4) *in vitro* studies. The diffusion of urea into the cell or out of the cell

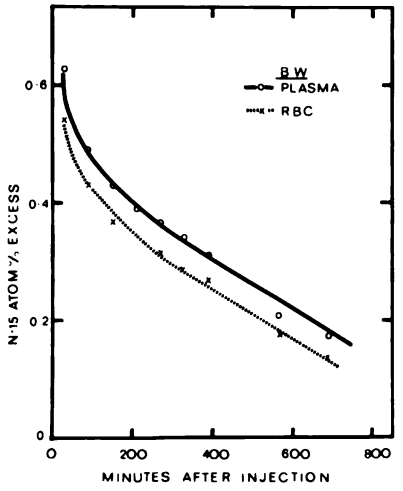

FIG. 1. Isotope concentration of plasma (O) and RBC (×) urea following intravenous injection of [^{15}N]urea in subject BW.

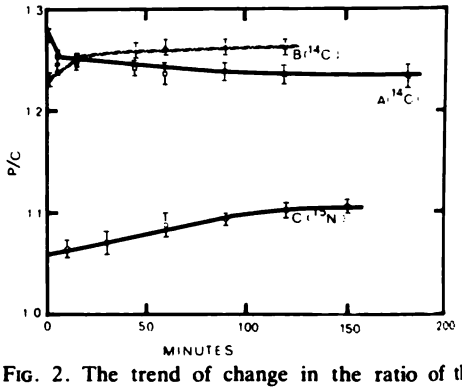

FIG. 2. The trend of change in the ratio of the tracer concentration (Mean±SEM) between Plasma (P) and RBC (C) with time (min) in *in vitro* studies: A: [^{14}C]urea, influx: n = 4. B: [^{14}C]urea, efflux: n = 2. C: [^{15}N]urea, influx: n = 4.

TABLE I. TURNOVER RATES OF UREA[a] FROM PLASMA (TRP) AND RED BLOOD CELLS (TRC) AND THE RANGE OF (P/C)[b] Ratios.

		First (10–180 min)			Second (180–570 min)		
Subject	Sex	TRP	TRC	P/C Range	TRP	TRC	P/C Range
JL-1	M	37.1 (0.82)[c]	32.4 (0.78)	1.170–1.087			
JL-2	M	80.2 (0.93)	78.1 (0.93)	1.191–1.167			
GR	M	18.9 (0.96)	11.8 (0.93)	1.259–1.187			
MS-1	M	28.4 (0.93)	26.5 (0.91)	1.106–1.080	12.1 (0.97)	14.2 (0.92)	1.080–1.120
MS-2	M	64.2 (0.99)	50.4 (0.96)	1.176–1.073	13.9 (0.99)	14.1 (0.94)	1.073–1.110
KK	F	25.9 (0.98)	25.7 (0.99)	1.154–1.138	18.8 (0.99)	19.3 (0.99)	1.138–1.170
BW	F	38.8 (0.95)	31.6 (0.90)	1.192–1.142	18.5 (0.98)	20.9 (0.96)	1.142–1.171
Mean		42.0	36.7		15.8	17.1	
±SEM		8.4	8.1		1.2	3.5	
P		<0.005	<0.005		<0.001	<0.005	

[a] The turnover rates (fraction/min) × 10^4 are the slopes obtained by the linear regression analysis of the logarithm of [^{15}N]atom % excess with time.

[b] The range of the ratio of [^{15}N]atom % excess between Plasma (P) and RBC (C) are included to show the trend during that interval.

[c] The numbers in brackets indicate the correlation coefficient ($-r$) obtained in fitting the experimental data by regression analysis.

fast and the steady state due to usional flow would have been even in the 1 min sample. The nt data represented mostly the ki- the urea in a different state. A decrease (−0.00021/min) in P/C the influx experiments and an in--0.00013/min) in the efflux experi- ith time were seen. Figure 2 also s the change in the P/C ratio from *itro* influx experiments ($n = 4$). It mphasized that no isotope effects ved in these studies. The measure- radioactivity in [^{14}C] experiments he amount of freely exchangeable ent in the respective compartments in the [^{15}N] studies the atom % dicates the relative amount of the the total urea (sp act). Since the ty in the chemical determination l difference in the concentration of ween plasma and red blood cells rger than the difference itself (2), pts were made in the [^{14}C] studies re separately the urea concentra- these micro samples. But on the d, in the [^{15}N] studies the simulta- asurement of 28 and 29 mass peak eads directly to the sp act [29/)].

ssibility for any systematic analyti- in our [^{15}N] studies was also ex- Any contamination of plasma in lead only to a lower P/C ratio and value will be still higher than ob- Deproteinizing, permutite and eatments, release of ammonia, and alyses were all carried out identi- plasma, cells, and blood. The pres- about 13% more free glutamine aragine) in cells than in plasma (2) rise to a slightly different amount i-labile" ammonia during the Con- sion procedure. This may account ximum P/C ratio of 1.003 which is e when one compares with our lues of 1.176 and 1.094 from *in in vitro* studies respectively. The entration of CO_2-NH_3 adduct (car- cid, carbamate, carbamyl phos- any, in cells will not influence our Urease blank determinations did any detectable (by mass spectrom- eter) ammonia nitrogen. The absence of urea enzymes in erythrocytes (9) ruled out the possibility of any cell urea production after separation of the two blood phases. A material balance was obtained between the sp act of whole blood and plasma or cells.

A concentration gradient promotes as isotope flux in our *in vitro* studies. The rate of transport of urea from the plasma into red blood cells may be represented by the equation:

$$d\{A\}/dt = -k'\{A\} + k''\{B\} \qquad (1)$$

where A and B are the concentrations of urea in plasma and cells respectively, k' and k'' are the "transfer constants". At equilibrium $d\{A\}/dt$ becomes zero. Thus,

$$k'/k'' = \{B\}_{eq}/\{A\}_{eq} = K \qquad (2)$$

On integrating for a time interval, t_1-t_2, and substituting with respective concentrations (10), equation 1 becomes:

$$-(t_2-t_1)\,(k' + k'') = ln\,[(\{A\}_2K - \{B\}_2)/(\{A\}_1K - \{B\}_1)]. \qquad (3)$$

Using the measured values of K (0.808 for influx and 0.791 for efflux), the "transfer constants" ($k' + k''$) were calculated for the 0–1 and 1–5 min intervals in the [^{14}C] experiments. The corresponding values (min^{-1}) of ($k' + k''$) were 4.042±0.026 and 0.170±0.008 for the influx studies and 4.548±0.035 and 0.066±0.020 for the efflux studies. In [^{15}N] *in vitro* studies K = 0.907 and ($k' + k''$) for 30–90 min interval was 0.023±0.004.

Discussion. When added to the blood [^{14}C]-labeled urea distributes itself between the cells and plasma in the same ratio of the freely exchangeable urea already present. About 97% of this distribution is attained in less than a minute and it takes about 2–3 hr to complete the equilibrium. Between equal volume of the components [^{14}C] radioactivity counts give directly the distribution ratio of free urea. This ratio at 1 min (1.277±.016, $P < 0.005$) closely follows that of water (1.288±.007, $P < 0.001$) demonstrating the identical concentrations of exchangeable urea per unit volume of either plasma water or cell water. One would expect this identity between these two phases which have almost no

permeability barrier. The analytical techniques followed in other studies (2-5) did not permit to distinguish this similarity in concentration. They have reported that the urea concentration in red cell water is higher than that in plasma water. This type of difference is observed by us in our [^{15}N] experiments where the concentration of the tracer with respect to the total urea (free and bound) was measured. The P/C ratio in our [^{15}N] studies should be one if the urea is present only in the free form. The observed significant deviation of this ratio from unity in both *in vivo* (1.176±.010, Mean±SEM) and *in vitro* (1.094±.011) studies shows clearly that urea is not distributed in the same freely exchangeable form between the blood compartments. The [^{15}N] atom % excess is always higher in plasma indicating that more bound urea is present in cells than in plasma. The P/C ratio is about 7% lower in the case of *in vitro* [^{15}N] studies. This appears to be a significant one since the pooled mean square analysis (11) of the data and the resulted *t*-distribution statistics gives P at <0.005. The small but significant difference may be due to loss of a substrate, similar to that of 2,3-diphosphoglycerate in the binding of oxygen by hemoglobin (12) resulting in less bound urea in cells. Conditions like pH, PCO_2 etc. were not controlled in the *in vitro* studies.

The fractional turnover rate of labeled urea from the plasma is not the same as that from the cells (Table I), indicating a different state of urea. In the initial period (up to about 180 min) there is a fast decay of the isotopic urea and the slope from cells is about 13% less than that from plasma (paired *t*-test $P < 0.02$). The P/C ratio is always greater than one, and there is a trend of its slow decrease with time (Table I). Thus in this period of equilibration there is a slow exchange and replacement of the bound cell urea by the labeled urea. In the second period (180-600 min) the decay of the isotopic urea is slowed down and the turnover rate from cells is about 8% higher than that from plasma. Now the P/C ratio slowly increases with time showing the slow replacement of the labeled bound cell urea. Our value of the fractional turnover rate $(15.8\pm1.2) \times 10^{-4}$ min^{-1}, of urea from the plasma agrees very well with the reported value $(15.5\pm1.1) \times 10^{-4}$ min^{-1} of Walser (13). No studies were done previously with the cells. Whether the [^{15}N] urea is injected (MS-1) or produced endogenously (MS-2), its decay pattern and distribution data between the two components of blood are almost the same indicating the indistinguishability of the source of marker urea.

The trend of decrease with time in the P/C ratio of the radioactivity in *in vitro* influx studies and a trend of increase in P/C ratio of the specific activity in the [^{15}N] *in vitro* experiments (Fig. 2) clearly demonstrate a slow influx of urea into the components of the red blood cells. These *in vitro* results and the absence of parallelism in the rate of clearance of labeled urea from the cells and plasma confirm that urea in human red blood cells exists both in a freely exchangeable form and in a relatively slowly exchangeable bound state. Plasma occupies a central position in the distribution and transport of urea. Both in the hepatic addition and in the renal extraction of urea from the plasma, the concentration in the red cell water is also perturbed but quickly adapted to the changing conditions. The similarity of the transfer constants for urea obtained in the first min of our influx and efflux *in vitro* studies suggests the operation of the same mechanisms in both directions. Following this initial rapid transfer of urea, the observed slowdown may be due only to the interaction with proteins.

Binding of a small molecule by a protein is the pivotal step in a host of biological functions including their transport throughout the vascular system. Significant quantities of urea are known to be bound to various proteins (3, 14). Hemoglobin is the most likely cell constituent that might form a urea complex. There may be a conformational accommodation, which merely stabilizes the complex and does not trigger a physiological process for either the protein or the ligand (15). Actually such a molecular adaptation to the relatively high urea concentration was found *in vivo* by elasmobranch hemoglobins (16).

When the equilibrium is shifted either by addition or extraction of urea, the bound urea becomes labile to maintain equilib-

Although the blood urea nitrogen in
'enous blood is lower than that in the
l blood by about 9% which corre-
; to the urinary excretion, there is no
nce between the arterial and venous
with respect to urea concentrations
cell water or plasma water (17). This
es that the perturbed equilibrium be-
the urea concentration of the red cell
and plasma water due to the overall
ıce of urea has already been restored
blood leaves the kidney. During
lialysis of uremic patients (18) when
tracellular urea concentration is con-
ɔly reduced, it takes about 15 hr post
s to attain the predialysis equilibrium
ɔn extracellular (plasma) and intracel-
muscle) urea concentrations. It does
ke this long to attain equilibrium
ɔn red cell water and plasma water
ut the low degree of hemolysis ob-
in some cases of dialysis may partly
to a delay in attaining equilibrium
ɔn the bound and free urea especially
ɔells.

distribution ratio of the freely ex-
able urea between equal volumes of
and cell is 1.277. The [^{15}N] atom %
ratio 1.176 (our [^{15}N] *in vivo* mean
represents the ratio of the ratio be-
the free urea and total (free and
) urea present in plasma and cell
rtments.

$$F_p/F_c = 1.277 \quad (4)$$

$$F_p + B_p)/(F_c/F_c + B_c) = 1.176 \quad (5)$$

F and B represent the amount of free
ɔund urea and the subscripts p and c
ɔnt plasma and cells respectively.

$$(F_c + B_c)/(F_p + B_p) = 0.921 \quad (6)$$

e the cell and plasma proteins have
ɔinding capacities for urea (3), their
tive concentrations 0.3298 g protein/
l and 0.07695 g protein/ml plasma
e related to the relative amount of
urea between the two compartments.

$$/B_c = 0.07695/0.32980 = 0.233 \quad (7)$$

hese relationships one can obtain $F_p/B_p)$, $B_p/(F_p + B_p)$, $F_c/(F_c + B_c)$ and $B_c/(F_c + B_c)$ ratios. It is calculated that about 96% urea in the plasma phase is freely exchangeable compared to only 82% in the cells. Thus in normal human blood (Hct. 45) about 10% of the urea is not readily available for free exchange but can be mobilized under specific conditions. In model simulations of urea transfer especially during dialysis (6, 7), this unequal distribution of urea between the two components of blood should be taken into consideration. Neglecting the possible differences between whole blood and plasma concentrations of urea and its rates of equilibration between red cells and plasma, Bass *et al.* (6) found that the calculated clearance of urea is 17% greater than the clearance calculated using whole blood levels and flow, or plasma levels and flow. This suggests the existence of a difference in the rate of removal of urea from the two components of blood which is confirmed by our observations.

Summary. The distribution kinetics of urea between plasma and the red blood cells of normal human blood have been investigated *in vivo* using [^{15}N] urea and *in vitro* using [^{15}N] urea and [^{14}C] urea. The partition ratio of free exchangeable urea between the cell and plasma is found to be the same as that of water distribution, showing the identical concentration (urea/ml water) of free urea in these two compartments of blood. However a significant difference (18%) is observed in the sp act ([^{15}N] atom % excess) between the urea present in the cell and plasma compartments of blood. This indicates that about one-fifth of the cell urea may not be in a freely exchangeable form. The fractional turnover rate of urea from the red blood cells is different from that of the plasma compartment suggesting the relatively bound state of intracellular urea and its slow mobilization.

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Ontogeny of Mink IgG, IgA, and IgM (40039)

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tian disease (AD) in adult mink is erized by marked hyperglobuline-lasmacytosis and immune complex ; and glomerulonephritis (1). The ammaglobulinemia and immune x disease in young mink infected in vith AD virus (ADV) was found to than that found in infected adult presumably because of decreased im-gical responsiveness in mink kits (2). more, mink kits infected neonatally DV frequently developed AD with l features rarely found in mink in-as adults (W. J. Hadlow, personal nication). Because immune respon-s is required for development of AD owledge about the ontogeny of im-in mink may help in elucidating the hysiology of AD in neonatal mink. al humoral immunity in mammals is iation of passively acquired antibody acental and milk transmission) and synthesized Ig by the host. The amount of maternal Ig transferred and after parturition varies widely mammals as does the particular Ig is transmitted (for review see Ref. present report contains information he transmission to and synthesis of A and IgM in young mink.

rials and methods. Animals. Sap-nink were obtained from a single herd free of AD and New Zealand abbits were obtained from a local . Serum or plasma was obtained rmal mink by cutting a toenail or by puncture. Blood was obtained by ating pre- and postnatal mink kits.

era. Antisera specific for mink IgG, d IgM were made in rabbits as previ-escribed (5), except that the antigen was absorbed by coupling it to Sepharose 4B (Pharmacia, Uppsala, Sweden) with cyanogen bromide (6).

Quantitation of Ig. Concentrations of mink Ig were determined by ring diffusion assay in which five lambda of serum were delivered into a 14-gauge needle hole made in antibody-agar covered glass plates. Appropriate concentrations of specific antibody and test serum were used to obtain suitable ring diameters. Concentration of protein in an unknown sample was calculated by comparing ring diameter (squared) with the dilution curve of the standard control serum. Isolated Ig of known protein concentration (Kjeldahl method) was used to determine concentrations of IgG, IgM and IgA in standard control serum (7). The molecular size of IgA in milk and young mink serum was not determined; for quantitation purposes this IgA was assumed to be similar in size to the adult serum IgA standard which was 12S (5).

[^{14}C] Tissue culture. Mink tissues [axillary, prescapular, and mesenteric lymph nodes, spleen, thymus, liver, ileum, and kidney] obtained at necropsy were promptly minced into 1 mm pieces that were incubated in culture tubes containing [^{14}C] isoleucine and [^{14}C] lycine (ICN, Irvine, CA) tissue culture medium and placed on a roller drum for 12 hr at 37° (8). After the cultures were frozen, thawed and centrifuged, the supernatant fluid was dialyzed extensively and then lyophilized. Presence of radioactivity in a particular Ig was examined by autoradiography in which the reconstituted [^{14}C] tissue culture supernatant fluid and normal mink serum carrier were added to a well of a 2% agarose covered slide and specific antiserum added to an adjacent well. Concentration of antiserum and carrier were adjusted so that a sharp precipitin line developed after 24 hr. The slides were then washed extensively for 4 days with

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PBS, dried, stained, and applied to Kodak contrast film for 7 days. Under the area of the superimposed agarose precipitin line [^{14}C]-labeled Ig was seen as a dark line on the developed film.

Results. Ontogeny of serum Ig. Serum levels of IgG, IgA and IgM were determined in blood obtained from normal sapphire kits exsanguinated at various intervals after birth (Fig. 1). The normal adult Ig values in Fig. 1 were obtained by examination of serum from eight 6-month-old male sapphires. Serum from a single kit was obtained on most days, although sera from two animals were examined on postnatal days 10, 20, 24, 31, and 50. IgM and IgG were detected within 24–48 hr after birth; concentration of IgM gradually increased over 128 days, whereas that of IgG rapidly attained adult levels (7–10 mg/ml) 8 days after birth. IgG levels then decreased to 1–2 mg/ml during the fifth week of life and before the kits were weaned on day 56. Serum IgA was not detectable until 39 days after birth and remained at low levels (0.01 mg/ml) until after day 128 (Fig. 1).

The young kits in Fig. 1 had been maintained until exsanguination with their mothers and presumably started suckling shortly after birth. One litter was examined which had not suckled; the parturient mother was maintained in a cage with a course screen bottom (1 × 2 in. mesh) and nest box without floor so that directly after delivery the kits fell through the screen out of the mothers' reach. Sera obtained from these four kits revealed no detectable IgA or IgM but an average IgG level of 0.56 mg/ml (0.35, 0.47, 0.67 and 0.73 mg IgG/ml). A serum pool also was obtained from one litter of fetal mink (1–2 weeks prepartum) and contained 0.1 mg IgG/ml without detectable IgA or IgM.

The high level of serum IgG in neonatal mink during the nursing period suggested its maternal origin. Twelve samples of colostrum and milk obtained 1–50 days after parturition were assayed for IgG, IgA and IgM (Table I). IgG was present in greatest quantity (mean 2.9 mg/ml), although small amounts (mean 0.17 mg/ml) of IgA were also detected. IgM was not present in any of the samples. Sera were obtained from

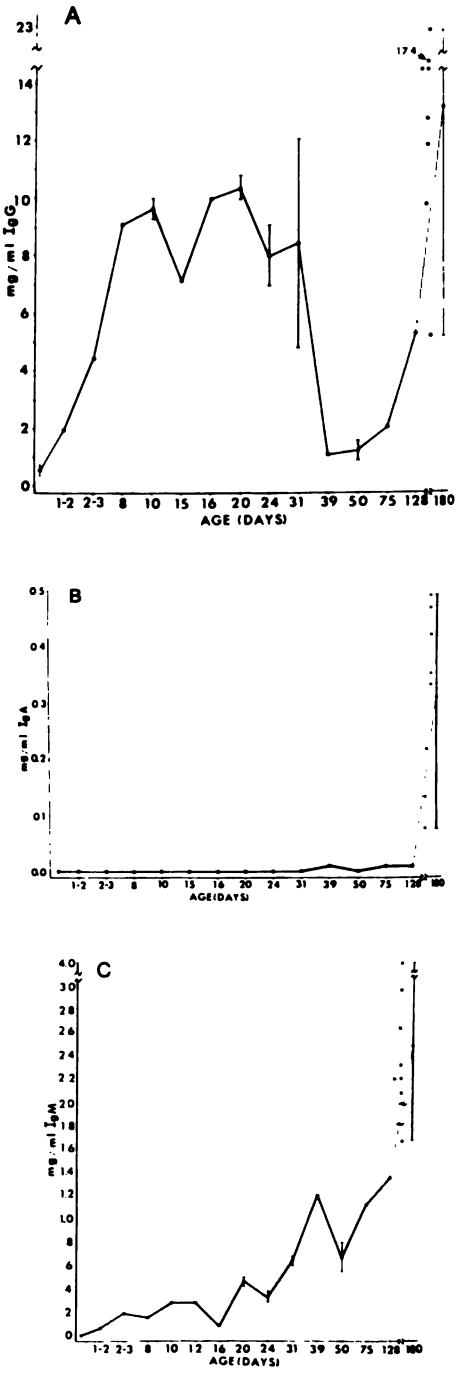

FIG. 1. Ontogeny of serum Ig in mink; mg/ml IgG (A), IgA (B) and IgM (C) shown at varic intervals after birth (day 0). Solid line connects me values. Range is shown by brackets. Adult individ values (dots) and means were obtained from sera eight normal male sapphire mink at 180 days of age

.E I. Ig LEVELS IN MILK AND SERUM OF LACTATING FEMALE MINK.

	IgG[a]	IgA[a]	IgM[a]
	2.9	0.17	0
	(1.0–6.3)	(0.04–0.5)	
	23.4	0.51	3.5
	(11.6–37.5)	(0.1–1.8)	(2.1–7.5)
×	14.5%	45.6%	0
:-	(4.4–25.6)	(27.7–84.0)	

age mean value in mg/ml and (range).
ve samples, days 1–50.
t samples, days 1–50.
t individually paired samples, days 1–50.

:tating females concurrently with the mple and the amount of serum Ig ermined. Calculations of milk/serum s for individual mink showed that, ıverage, relative IgA secretion was d greater than IgG secretion; that n IgA level in milk was 45.6% of evel, whereas mean IgG concentra- nilk was 14.5% that of serum (Table significant trend of change in Ig ıf milk and serum with time were though the highest levels (mg/ml) of nilk (0.5 and 0.34) were on days 39 and the lowest on days 1 and 2 ıd 0.08). The highest IgG (5.0 and ml) levels in milk were from mothers d the highest IgG (32.2 and 37.5 espectively) levels in serum.

Synthesis of mink Ig. Lymph nodes ɔular, axillary and mesenteric), thymus, liver, and intestine from ınguinated kits (Fig. 1) were assayed ynthesis by [^{14}C] tissue culture. IgG M synthesis were consistently de- n lymph node and spleen in all kits 28 days of age. Smaller, but yet ve amounts of the IgG and IgM is also were found in thymus and kits up to 20 days of age. The first ive synthesis of IgA, however, was d in spleen of a 75-day-old kit and spleen, lymph node, and particularly ine of mink 98 and 128 days old.

ssion. Adult levels of IgG were n sera of kits from the first to the veek of life, presumably because of l Ig transferred by milk (9, 10). gG concentration then fell precipi- luring the fifth week of life and before weaning on day 56. Substantial amounts of IgG were present in milk throughout this interval, so the IgG decrease in kit serum occurred either because the capacity for its intestinal absorption decreased, as occurs in mice (11), or perhaps because suckling had essentially ended at this time. Sera of kits also contained autologous IgG during the neonatal period, as IgG synthesis was detected consistently from the first day of birth. The small amounts of IgG detected in fetal and neonatal mink prior to suckling could be of autologous and/or maternal (transplacental) origin. Kits also were synthesizing IgM at birth; the kinetics of increase during neonatal period and absence from milk indicate that kit serum IgM originates within the host.

In comparison to IgG, mink milk contained relatively small amounts of IgA, as also occurs in ruminants (12). However, when compared with milk-serum ratios of IgG (Table I), a threefold advantage of IgA secretion was found. The lack of detectable IgA in suckling kits may be explained by the small amount of IgA in milk. But mink kits, like suckling mice (13), may be unable to absorb milk IgA. Furthermore, little IgA of host origin was present, as IgA synthesis was not detected in young mink until they were more than 2 months old and significant serum levels were not achieved until after 4 months of age. Thus, ontogeny of mink IgA is similar to the slow maturation seen in other mammals (14). Also, the predominant synthesis of IgA by gut-associated tissues in young and adult normal mink (7) is similar to that in other animals (14).

Studies on the kinetics of serum Ig changes in adult mink after infection with ADV have shown that elevation of IgA levels was frequently preceded by increases in IgG levels (7), similar to serum levels in ontogeny. However, in sapphire mink with terminal AD, the serum Ig with greatest relative increase frequently was IgA, although AD resistant pastel mink showed minimal serum IgA increase (7). The question arises whether IgA-anti-ADV is an important component for development of AD pathology. Future experiments will determine if a minimal IgA-anti-ADV response

in sapphire kits infected with ADV can explain the observed mild disease in these young mink.

Summary. Serum levels and [^{14}C] tissue culture synthesis of IgG, IgA and IgM were determined in pre- and postnatal normal mink. Small amounts of IgG were found in serum from fetal and neonatal mink prior to suckling. This could represent maternal Ig (transplacental) or could be autologous Ig as IgG synthesis was found in day old kit spleen and lymph node tissues. The suckling kit achieved adult levels of IgG (7–10 mg/ml) 8 days after birth although IgG then decreased (before weaning) to 1–2 mg/ml during the fifth week of life. Generous amounts (mean 2.9 mg/ml) of IgG were found in mink milk throughout the nursing period. Small amounts of IgA (mean 0.17 mg/ml) were also found in mink milk, although IgA was not detectable in kit serum until 39 days of age and IgA synthesis in [^{14}C] tissue culture was not found in tissues from kits less than 75 days of age. IgM was not detected in milk, although [^{14}C] tissue culture synthesis of IgM was consistently found 1–2 days postpartum and serum levels gradually increased during neonatal development.

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Related Development of Pulmonary Antioxidant Enzymes in the Rat[1] (40040)

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pulmonary antioxidant defense
ıisms, superoxide dismutase and the
ione system, have been postulated
er varying degrees of protection
oxygen (1-5), ozone (6), nitrogen
: (7), and paraquat (8, 9) induced
)xicity. Oxidant-induced lung injury
rally thought to result from the cyto-
ction of the free radical superoxide
and other highly reactive intermedi-
ıch as hydroxyl radical, peroxy radi-
l hydrogen peroxide (1-10). Super-
lismutase (SOD) is believed to dimin-
dant-induced lung injury by catalyz-
: dismutation of superoxide radical
ıe enzyme catalase (CA), which can
luded in the superoxide dismutase
. sequesters the hydrogen peroxide
l from the dismutation reaction (10).
ıtathione system includes glutathione
lase (GP), which is thought to limit
)eroxidation initiated by superoxide
that has escaped dismutation (11).
ed glutathione (GSH) is utilized as a
)r in this reaction. The intracellular
f GSH can then be replenished by
ıione reductase (GR).

ıs long been recognized that newborn
:onatal animals are more tolerant to
:ic pulmonary effects of oxygen than
(4, 12, 13). We have previously
gated the mechanism of this age-de-
ıcy of oxygen-induced lung toxicity
ıve observed that the relative resist-
)f the neonatal animal to hyperoxic
ıjury is associated with an increase in
:ivity of their pulmonary antioxidant
e systems as compared to the adult (4). Recently Kehrer and Autor (14) have examined the activity of various rat pulmonary glutathione system enzymes as a function of age. In the present study, we have further examined the development of these pulmonary antioxidant defense systems in an expanded age range in rats including premature rats and rats between 15 and 70 days old in an attempt to gain additional understanding of the mechanism responsible for age-related susceptibility to oxygen-induced lung injury.

Materials and methods. Procedure in animals. Animals used for these studies were Sprague-Dawley albino rats of varying ages (2 days premature to 62 days old) bred and raised in the Animal Care Facility at The University of Iowa and Sprague-Dawley albino rats (70 days old) obtained from Biolab Corporation (St. Paul, MI). All rats were sacrificed by decapitation on the same day and their lungs were carefully perfused with ice-cold isotonic phosphate buffer via the pulmonary artery (4). Lungs were trimmed to remove extraparenchymal, tracheal-bronchial and vascular tissue and were homogenized for 2 min in a 1:15 (W:V) dilution with ice-cold, 0.005 M, phosphate buffer, pH 7.8, using a Sorvall Omnimixer (Ivan Sorvall Inc., Newtown, CO). Lung tissue from two to 14 premature or young animals (less than 50 days old) were pooled for purposes of analysis. Rats greater than eight days old were separated according to sex and were analyzed accordingly. Superoxide dismutase activity and GSH, deoxyribonucleic acid (DNA) and protein levels were analyzed in the lung homogenate. Glutathione reductase, GP and CA activities and protein concentration were determined in the 15,000*g* lung supernatant. Attempts were made to perform a single biochemical assay on all the samples in a random fashion on the same day.

Biochemical assays. Lung SOD activity

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was determined by inhibition of ferricytochrome *c* reduction by xanthine and xanthine oxidase (15). GR activity was determined by following NADPH disappearance (16); GP activity by the method described by Lawrence and Burk (17) using cumene hydroperoxide as substrate; and, CA activity by the method of Holmes and Masters (18). Assay of GSH level utilized the modified method of Klotsch and Bergmeyer (19) by Delucia *et al.* (20). DNA was determined using Richard's modification of the diphenylamine reaction (21) and protein concentration by the modified Lowry's assay (22).

Results. No significant differences were found in the various enzyme activities and GSH levels between male and female rats. To simplify the figure presentation, data from male and female rats of the same age group were therefore combined. Each value in the figures (1–5) represents mean ± SE of two to five individual samples for each age group. A similar pattern with age was observed in pulmonary SOD activity (Fig. 1) and GSH level (Fig. 2), whether the activity level was expressed as per mg protein or per mg DNA in lung homogenate. Pulmonary SOD activity and GSH level of 70-day-old rats were approximately 200% of that found in neonatal rats (0–21 days old) when expressed as per mg DNA and 150% of that found in neonatal rats v expressed as per mg protein.

Pulmonary GP (Fig. 3) and CA (Fi activities appeared to increase from 2 premature to birth and then subsequ decrease to approximately the original level by age 8–12 days. For SOD (Fi and GR (Fig. 4), this pattern of en activity was less obvious. As with SOD and GR activities showed a progressiv crease with age from day 12 to 70. C contrast to all the other enzymes and C showed a progressive decline in ac from day 12 to 70. Activities of GP. and CA in 70-day-old adult rat lung

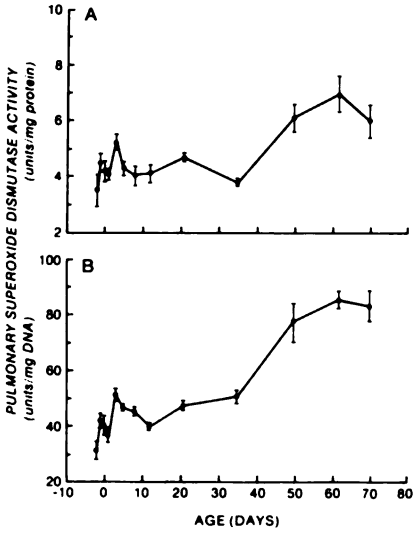


FIG. 1. Pulmonary superoxide dismutase (SOD) activity at different ages in the rat expressed as units activity per mg protein (A) and per mg DNA (B).

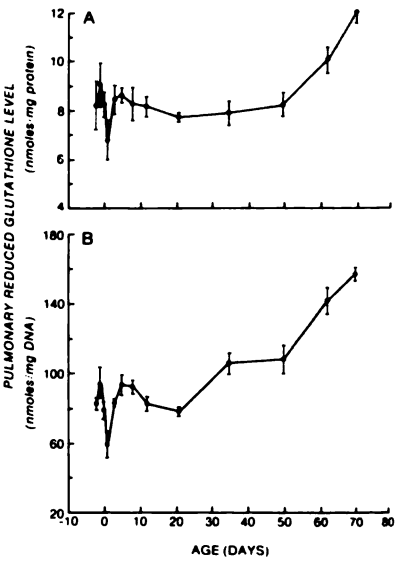


FIG. 2. Pulmonary reduced glutathione (level at different ages in the rat expressed as n per mg protein (A) and per mg DNA (B).

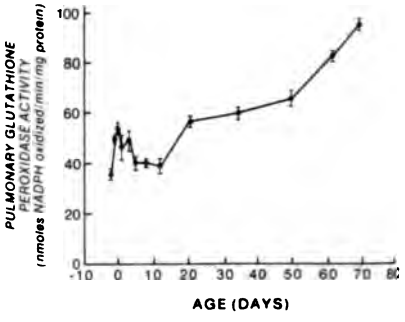
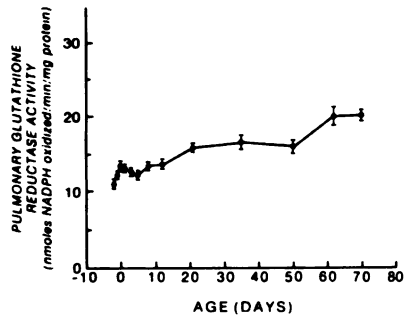
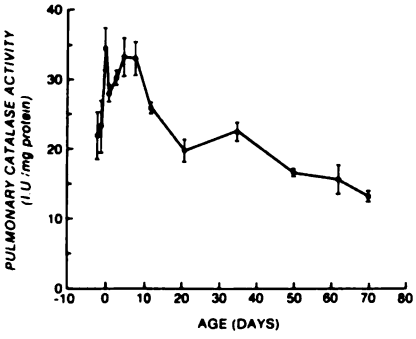


FIG. 3. Pulmonary glutathione peroxidase activity (nmoles NADPH oxidized/min/mg prot different ages in the rat.



. 4. Pulmonary glutathione reductase (GR) ac- (nmoles NADPH oxidized/min/mg protein) at nt ages in the rat.



. 5. Pulmonary catalase (CA) activity (I.U./mg n) at different ages in the rat.

oximately 200%, 150% and 50%, re- ively, of that found in neonatal rat

scussion. Previous studies have shown elated changes of various pulmonary mes (23, 24). In the present study, the of activity of all the pulmonary antiox- t enzymes studied (Figs. 1, 3-5) were r at 2 days before term than at birth. increase in activity from 2 days before to birth may serve an important func- in preparing the transition of the lung l a relatively hypoxic environment *in*) to the much richer extrauterine oxy- environment. Fluctuations in enzyme ities and GSH levels were observed een birth and 5 days of age. The expla- n for these fluctuations is not known t could be a consequence of the multi- of environmental and physiological ges which are known to be occurring ig this time.

om day 12 to 70, SOD, GP, and GR ities and GSH levels generally showed a gradual increase with age. In contrast to the other enzymes studied, pulmonary CA activity was less in the older rats compared to the younger animals (Fig. 5). Similar findings were reported by Kehrer and Autor (14) in pulmonary GR and GP activities but not with GSH levels in developing rat lung. The increase in pulmonary GSH levels with age observed in the present study may be attributed to the different analytical techniques employed in these two studies.

Young adult rats (70 days old), who have been repeatedly shown to be more susceptible to oxidant-induced lung damage (4, 12, 13) have nevertheless a 1.5- to twofold greater SOD, GP, and GR activities and GSH levels than that of neonatal animals. Human adult lung has similarly been found to have a greater SOD activity than fetal and newborn lung (25). This apparent paradox may be explained in part by our recent findings that neonatal rats, in response to high oxygen exposure, showed a rapid and significant increase in pulmonary SOD, GP, GR, CA activity, and GSH levels (4, 5, 25, 26). In these studies, adult rats, although possessing greater baseline lung activity levels of SOD, GP, GR, and GSH (Figs. 1-4), failed to respond with any increase in these enzyme activities or GSH levels in response to a toxic oxygen challenge. The lower pulmonary activity of CA in adult rats may be extremely critical and further contribute to their greater susceptibility to oxidant-induced lung damage.

On the basis of this study and previously published work (1-10, 25, 26), one could speculate that the ability of an animal to tolerate oxygen-induced lung damage is governed by a delicate balance between the generation of cytotoxic agents and the various pulmonary defense capabilities. The intracellular production of cytotoxic agents is believed to be increased during exposure to high concentrations of oxygen (27). The ability of the lung to offset this challenge would be determined not only by the total activities of pulmonary antioxidant enzymes, but also by the capability of the lung to increase its antioxidant enzymes in response to hyperoxia and the proximity of these protective systems to the site of generation of cytotoxic agents.

Summary. The age-related development

of the activity levels of the pulmonary antioxidant defense systems: superoxide dismutase, reduced glutathione, glutathione peroxidase, glutathione reductase and catalase, were examined in the rat. All the pulmonary antioxidant enzymes studied showed a lower activity at two days before term than at birth. From day 12 to 70, with the exception of catalase, these enzymes and reduced glutathione generally showed a gradual increase in activity levels with age. Pulmonary catalase activity, however, was lower in the older rats compared to the younger animals. No significant differences were found in the various pulmonary enzyme activities and glutathione levels between male and female rats.

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IOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 297-300 (1978)

sue Cholesterol Storage: The Effect of Essential Fatty Acid Deficiency[1] (40041)

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of essential fatty acid[2] defi-
rat is associated with increased
ster content in a number of
issues, most notably skin (1),
and ovary (3). In skin, accu-
sterol esters may account in
abnormal keratinization, tail
scaliness which represent the
tifying features of EFA defi-
has been suggested that the
tent of skin cholesterol esters
ulation of cholesterol esterify-
vith simultaneous inhibition of
ster hydrolase activity, all as-
lack of prostaglandin synthesis
nic acid (5). Unlike skin, the
ovary normally contain large
holesterol esters. In these tis-
: of the preferred substrate,
rachidonate, for steroid hor-
is induces utilization of other
ters which normally serve only
apacity (2, 3).
demonstrated that rat adipose
ntains cholesterol esters (6–8),
ably may be hydrolyzed enzy-
mobilization (9). This tissue
synthesize prostaglandins *in*
sential fatty acids (10). Such
ell as the results obtained in
es mentioned above prompted
e the effect of essential fatty
:y on the storage of free and
lesterol in isolated fat cells of
eliminary report has appeared

wenty male weanling Sprague-
ree weeks of age were divided
ary groups as shown in Table
rats received a fat-free diet (experimental I) or a control diet containing corn oil (control I). Group II experimental rats were fed hydrogenated coconut oil in order to produce a more rapid and severe form of EFA deficiency than the fat-free group (4). Control group II received corn oil together with hydrogenated coconut oil. Thus, all animals except the fat-free group received 7% lipid, 20% protein and equivalent amounts of carbohydrate. In addition to the dietary components listed in Table I, all diets contained 4% fiber as cellulose, 4% salt mix, 0.7% vitamin mix and 0.3% choline chloride. All of these semi-synthetic diets were provided *ad lib* for a period of 16 weeks.

Following decapitation of all animals, isolated fat cells were prepared from epididymal adipose tissue by the method of Rodbell (12) and both cells and plasma were extracted using an isopropanol–Zeolite system (13). Cell size was calculated as previously described by us (14) and others (15). Free and esterified cholesterol from extracted adipocytes were separated using lipophilic Sepahdex LH-20 gel filtration according to a method recently developed in our laboratory.[3] The Student *t* test was utilized to determine significant differences between means (16). All statistical analyses were done using a Wang Model 500 Programming Calculator (Wang Laboratories, Inc., Tewksbury, MA).

Results and Discussion. Retardation of animal growth has been used in early studies on EFA deficiency both as a criterion for the state of deficiency and in bioassays for determining the essentiality of various fatty acid derivatives (17, 18). As shown in Table II, both groups of essential fatty acid deficient rats in the present study also showed

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rant No. AM19995.

1: essential fatty acid (EFA).

[3] Krause, B. R. and A. D. Hartman. Submitted for publication.

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TABLE I. COMPOSITION OF CONTROL AND ESSENTIAL FATTY ACID DEFICIENT DIETS.

Group	Lipid	Protein	Carbohydrat
I			
Control	7% corn oil	20% casein	64% sucrose
Experimental	zero	20% casein	71% sucrose
II			
Control	2% corn oil 5% HCO[a]	20% casein	64% sucrose
Experimental	7% HCO	20% casein	64% sucrose

[a] Hydrogenated coconut oil.

TABLE II. AUTOPSY DATA FROM RATS FED CONTROL AND ESSENTIAL FATTY ACID DEFICIENT DIET

Group	Body weight (g)	Fat pad weight (g)	Cell size[b]
Control I	503.6 ± 25.1	7.1 ± 0.7	73.4 ± 4.(
Control II	500.4 ± 25.3	6.5 ± 0.5	59.9 ± 6.7
Pooled control	502.0 ± 16.8	6.8 ± 0.5	66.7 ± 6.2
Experimental I	383.6 ± 10.1[c]	5.4 ± 0.5	50.7 ± 5.7
Experimental II	401.6 ± 8.2[c]	6.2 ± 0.8	50.1 ± 5.9
Pooled experimental	392.6 ± 6.8	5.8 ± 0.4	50.4 ± 5.7

[a] Values are means ± SE of 6 rats/group.
[b] Cell size is expressed as μg triglyceride/cell × 10^{-2}.
[c] Significantly less than respective control at 5% level.
[d] Significantly less than the pooled control values at 5% level.

significantly less weight gain during the feeding period than respective control animals. The wet weight of epididymal fat pads in the EFA-deficient groups showed an average nonsignificant decrease of 16%, while mean cell size differed by 24% when compared to controls. However, differences in cell size between experimental and control rats did reach statistical significance when all control rats were pooled and compared to all EFA deficient animals as a whole (Table II). These tendencies toward change in adipose tissue triglyceride storage, which may largely contribute to the body weight retardation, are consistent with the original description of EFA deficiency by Burr and Burr in which it was reported that "storage fat can be almost entirely eliminated" when young rats are deprived of fat for several months (19). Such decreases in cell size were also not surprising in view of the fact that others have reported increased *in vitro* lipolysis in EFA-deficient rats (10, 20).

As expected, EFA deficient rats as a whole had significantly lower plasma total cholesterol levels than controls (Table III). This agrees with the work of Alfin-Slater *et al.* (21). The significant decrease in plasma cholesterol esters, resulting in an increased percentage of free cholesterol, has also been reported and has been ascribed to dec liver cholesterol esterification (22).

On the other hand, the levels o cholesterol in adipocytes, as shown in IV, were higher than those previou ported for Sprague-Dawley rats of body weight fed normal rat chow (7, is possible that this is due to the se thetic nature of the diets, as well as high sucrose content, both of which ir plasma, liver, skeletal muscle, and a tissue lipid levels (7, 18, 23). Althou size was not different between experi groups (Table II), deficient animals ing lipid in their diets (experimental significantly less adipocyte total as free cholesterol compared to rats re no dietary fat (experimental I). This seem to support the contention that eride content or cell size is not th determinant of adipocyte cholestero mulation (14, 24). The largest adip did not have the greatest content of cholesterol, which also suggests disso of cholesterol storage from that of tri ide.

Adipocyte total cholesterol in conti was approximately 36% cholesterol These data for the percent of total terol as esters agree most closely wi

'ABLE III. Effect of Essential Fatty Acid Deficiency on Plasma Cholesterol Levels.[a]

Group	Free cholesterol	Cholesterol esters	Total cholesterol	% Free cholesterol
ıtrols	75.5 ± 5.5	71.4 ± 6.5	145.9 ± 11.1	51.2
ɔerimentals	73.9 ± 6.8	45.2 ± 3.8[b]	119.2 ± 10.5	61.9[b]

essed as mg/dl, $n = 4$ for each group.
ficantly different from control value at 5% level.

BLE IV. Effect of Essential Fatty Acid Deficiency on Adipocyte Cholesterol Storage.[a]

Group	Free cholesterol	Cholesterol esters	Total cholesterol	Percent cholesterol esters
·ol I	1353.9 ± 132	779.5 ± 81	2133.5 ± 173	36.7
·ol II	1195.5 ± 75	680.7 ± 56	1876.2 ± 128	36.2
rimental I	2011.8 ± 100[b]	847.9 ± 238	2859.8 ± 329[b]	29.9[b]
rimental II	1327.7 ± 214[c]	673.9 ± 151	2001.7 ± 363[c]	31.5[b]

essed as $\mu g/10^6$ cells ± SE, $n = 5$ for all groups.
ficantly different than respective control at 5% level.
ficantly less than experimental I, $P < 0.1$.

ıs *et al.* (6) who found that isolated
; of 400 g rats contained 24% of
olesterol as esters. Other laborato-
8) have reported much lower per-
er values. As discussed previously,[3]
ɔcrepancies arise most likely because
ıical difficulties inherent in determi-
ɔf free and esterified cholesterol in
ɜence of larger quantities of neutral
problem unique to adipose tissue.
ɔse tissue does not appear to re-
ɔ EFA deficiency like other periph-
ues. A significant increase in adipo-
:e and hence total cholesterol oc-
in rats deprived of dietary lipid,
esulted in a significant decrease in
:entage of total cholesterol as choles-
ɜters (Table IV). Stability of the
te ester pool under conditions of
ɜficiency was apparent despite the
ıced alterations in triglyceride (25)
bohydrate metabolism (26) known
r in EFA deficiency. A probable
ism for increased free cholesterol
in fat cells may be related to the
:d percentage of free cholesterol in
of EFA rats also reported in this
ır to changes in membrane structure
ılteration of phospholipid fatty acids
. deficiency (17). More work and
the use of other models are re-
o reveal the regulatory mechanisms
ing cholesterol fluxes in adipose tis-

Summary. Essential fatty acid deficiency was induced in one group of rats by feeding a fat-free diet, and in another group by supplying hydrogenated coconut oil. All deficient animals had significantly reduced weight gain and tended to have decreased epididymal fat cell size compared to controls. Rats in the fat-free group had significantly more adipocyte free cholesterol than control animals, but no significant alteration in stored free sterol occurred in rats made essential fatty acid deficient with hydrogenated coconut oil. Between experimental essential fatty acid deficient groups, the fat-free group had greater quantities of adipocyte free, and hence, total cholesterol. No alterations in ester content due to essential fatty acid deficiency could be demonstrated, although the percentage of total cholesterol as esters decreased significantly in both experimental groups. It is suggested that adipose tissue responds differently than other peripheral tissues to essential fatty acid deficiency.

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ole of Dextran Sulfate in Increasing the CFU_c^- Concentration in Dog Blood (40042)

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ıotent as well as committed stem
: present in the peripheral blood of
ls, although in rather small number
heir presence in the circulation may
indicative of the role they play in
ıtenance of hemopoietic homeosta-
ıgst the various sites of hemopoiesis
out the skeletal system. In the hu-
l other large mammals, a quantita-
ermination of the pluripotent stem
ulation in the blood is still a problem
lved; we must still content ourselves
: determination of the presumably
:ytic-committed stem cell, using the
Uc (colony forming unit in culture)
eveloped by Bradley and Metcalf
: number of CFUc appears to be a
od indicator for the presence of
ent hemopoietic stem cells in the
f dogs, as evidenced by the fact that
ietic recovery in the marrow of le-
radiated dogs is proportional to the
of blood CFUc transfused (1). In
ıstances, mononuclear leukocytes
including CFUc, had been col-
rom donor dogs via an arterio-ve-
ıunt using the IBM Experimental
:ell Separator. A 5-hr leukapheresis
:essary to collect 25 × 10^9 leuko-
mong these being about 8 × 10^9
vhich, in turn, contained some 0.5
FUc.

der to increase the yield of MNC,
particular, of CFUc from a single
eresis, dextran sulfate (DS) was ad-
ed iv shortly before the onset of
eresis. In previous studies, Ross et
shown that various polyanions, in-
DS, are able to increase the number
: in the blood of rats and of other
ls (6, 7). Thus, it was the objective of this study to examine in dogs the effects of administering different doses of DS on the blood levels of leukocytes, especially of MNC and of CFUc, to provide a basis for attempts to increase the yield of CFUc collected from the peripheral blood.

Materials and Methods. Two healthy beagles were used in this study, about 2 years of age and weighing 16 kg—one was male and the other female.

An anterio-venous shunt was surgically inserted between the carotid artery and the jugular vein on one side. This shunt was thoroughly cleansed (flushed with heparinised saline) twice a day to prevent blockage by fibrin buildup. It could generally be maintained free-flowing for 3–4 weeks. Antibiotics were administered only for the first few days following the operation while the wound healed.

Dextran sulfate (DS) is a synthetic polyanion of strong negative charge, composed of glucose units in a helical chain and with SO_4 groups. It was prepared for these experiments by esterifying pure dextran (m.w. 40,000, Merck) with chlorosulfonic acid, after the recipe used by Ricketts (8). On each glucose unit of dextran are three hydroxyl groups, any one or all of which may be substituted for by a sulfate group; in this case, an average of 2.3 sulfate groups were found per glucose unit, as indicated by a sulfur content of 17%. The product was neutralized, extensively dialysed against distilled water, then freeze-dried, powdered, and stored in air-tight vials at 4°. Just prior to administration, the appropriate amount of DS was dissolved (readily) in 20 ml of sterile saline solution (0.9%). It was injected over about 20 sec into the venous side of the shunt.

As can be derived from Fig. 2, each of the two dogs received on two separate occasions, saline injections (S) as a control. On four other separate occasions, 10 mg

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DS/kg body weight (D) were given. In two further experiments, 15 mg DS/kg body weight (D') were given. Between one experiment (lasting 7 hr) and the next, 2–3 days were allowed to elapse in order to stabilize the blood cell levels.

The number of CFUc in the peripheral blood, as withdrawn from the arterial side of the shunt at various intervals after administration of DS, was determined by using a modified agar culture technique (4).

The levels of leukocytes (mononuclear and polymorphonuclear) and thrombocytes in the peripheral blood were also recorded, along with the hematocrit.

Results. Figure 1 illustrates the findings for the two different doses of DS and for the saline control. The data from all experiments in the two dogs were pooled for each treatment schedule.

There was no effect caused by the administration of *saline* alone, either on leukocyte numbers or on CFUc, over the 7-hr period of observation. However, after the administration of *10 mg DS/kg* body weight, within 3 hr there was a slight rise in polymorphonuclear cells (PMN), a doubling of MNC, and a sevenfold increase in CFUc (from 100 to 700 CFUc/ml blood). After the administration of *15 mg DS/kg* body weight, there was an increase in PMN of about 40% over the preinjection values. The MNC showed an increase of about 100%, double the preinjection value. The blood CFUc experienced a tenfold increase in number, from about 200 to about 2000 CFUc/ml blood. The standard error for the 3-hr mean CFUc value was fairly large, due to the relatively high values observed in the first experiment (21-fold increase) as compared to the values in the second experiment (about fivefold increase), for both dogs.

The MNC population following DS administration tends to maintain the high level beyond 3 hr, as does the PMN population. On the other hand, the CFUc population experiences a decrease after the 3-hr peak. It should also be noted that the increase in CFUc over the first 3 hr is proportionally much greater than the increase in MNC, indicating that the CFUc concentration is controlled by mechanisms other than those controlling MNC and PMN levels. Normal values for leukocytes and for CFUc are regained within 24 hr.

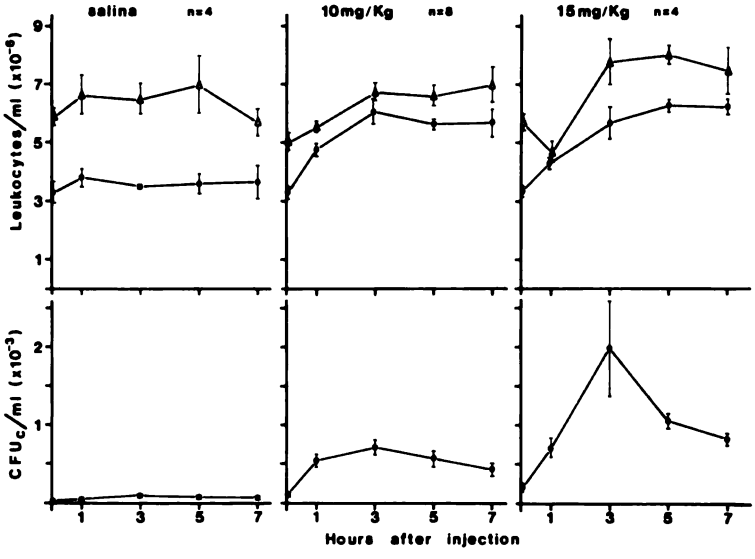

FIG. 1. Effect of dextran sulfate administration i.v. on blood leukocytes in two dogs. (a) Saline only (0.9%): $n = 4$. (b) Ten milligram DS/kg body weight (eight separate experiments, four with each dog). (c) Fifteen milligram DS/kg body weight (four separate experiments, two with each dog). Each point represents the mean; the bars indicate the standard error of the mean. — △ — PMN; — ● — MNC; — ○ — CFUc.

It is of interest, however, to note that the repeated administration (2-3 days between doses) of dextran sulfate (10 or 15 mg DS/kg body weight) results in an increase in the basic concentration of CFUc in blood (Fig. 2).

For the two dogs, the basic CFUc level (preinjection level) increased from about 65 to 230 CFUc/ml blood, a fourfold elevation from before the first dosage until 24 hr after the last. In contrast, other blood parameters remained essentially unaltered. MNC and PMN levels were unaffected. Thrombocytes displayed a slight tendency to decrease, declining to about 60% of their initial day 0 level, while the hematocrit decreased by 10%; these changes are considered to be mainly a consequence of the experimental procedures, namely the sampling.

Since it has been found by other investigators (9, 10) that the number of leukocytes and CFUc increase after injection of endotoxin, the dextran sulfate employed in these experiments was tested for endotoxin contamination by the commercially-available Limulus Assay (Associates of Cape Cod., Inc., Woods Hole, MA). Standard endotoxin from E. coli (Westphal method; code 0127:B8) was used as a positive control for a quantitative determination; it produced a positive reaction down to 0.5 ng/ml. Dextran sulfate (15 mg/ml) gave essentially a negative reaction; if endotoxin was present as a contaminant, it must have been in a quantity considerably less than 0.5 ng/ml

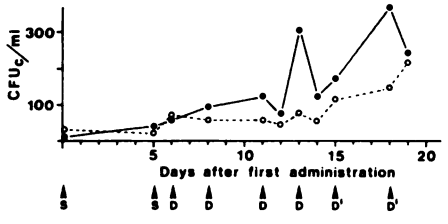

FIG. 2. CFUc concentration in peripheral blood during the 3-week period of study; effect of repeated doses of dextran sulfate on both dogs. Values shown were taken just before each administration of DS, or on another day. Note the tendency for the CFUc to increase progressively as further doses of DS are experienced. S = saline only (0.9%); D = 10 mg DS/kg body wt.; D' = 15 mg DS/kg body wt.

which, according to the literature, would have no effect in any *in vivo* system.

Discussion. Previously, many polyanions (especially polysaccharide polysulphates, such as the heparinoids) have been observed to increase the number of MNC in the blood of mammalian species (6, 11-13). With these observations in mind, we asked ourselves the question, if stem cells (either committed or noncomitted) are part of the blood MNC population, might not polyanions be able to increase the number of stem cells in the blood too? This turned out to be indeed the case, but the CFUc increased even more than expected. While the MNC increased twofold after 10 mg DS/kg body weight, the CFUc increased some sevenfold, and after 15 mg/kg, some tenfold.

This would suggest that, although DS also affects the lymphocyte count, it is particularly effective in raising the number of CFUc in the blood within 3 hr of iv administration by 7-10 ×, depending on the dose administered.

At the present time, the mechanisms that are effective in increasing the CFUc concentration in the blood after dextran sulfate administration are far from clear. In principle, one must consider actions that lead to a "mobilization" of cells from extravascular sites by allowing increased access to the blood and thereby increasing the concentration in the blood. Along the same lines would be the argument that an increased number of CFUc in the blood could be expected if there were a marginal pool from which there was a shift of cells into the circulating pool under the influence of dextran sulfate. However, one must also entertain another possibility. If the blood transit time of blood stem cells were altered, were in effect prolonged, then one would also quickly observe an increase in the number of circulating CFUc. Bradfield and Born (14) have made a detailed investigation of the interference with lymphocyte recirculation by heparin and DS in mice and rats. This phenomenon may also apply to stem cells. In this context, it is of interest to consider ways and means by which polyanions may act on cells. Some investigations indicate that DS and other related poly-

anions may have an affinity for the membrane of target cells (15). They may in fact be attached to the cell membrane in the form of small granules (16). Reversible binding of the polyanions to the negative cell surface, perhaps by means of bridges of Ca^{2+} ions (18), would increase surface negativity. Thus, the electrophoretic mobility would be increased (17, 19). This increased negativity hinders adhesion of cells to one another as well as to the endothelium (17, 20). Such an action could result in an increased release rate from the tissues where they were lodged, and/or in an inability to "home" in these or other tissues. In this context, it has been further observed that incubation of cells with DS decreases membrane deformability, as demonstrated by the loss of the ability to adhere to glass surfaces (21). All of these factors might be involved in decreasing the anchorage of the cells outside the vascular channels and in making them more mobile for entry into the blood at the appropriate sites (as yet poorly understood) in the bone marrow.

In addition to this mechanism (or as an alternative), one must certainly consider the possibility that the CFUc increase is not due to an increase in the release rate of CFUc from extravascular sites, but rather to a decrease in their disappearance rate from the blood. If the CFUc transit time through the blood were somehow prolonged, perhaps because the CFUc were experiencing difficulties in adhering to the endothelial wall, this could give rise to the observed effect. These hypotheses can be tested by future experiments on the basis that the polyanion effect can be readily and completely neutralized by the addition of protamine (a polycation) in both the *in vivo* and the *in vitro* situations (7, 17). A final word should be said about the toxicity of dextran sulfate. It has been found that toxicity increases with increasing molecular weight but that DS of low molecular weight, such as 10,000, is no more toxic than heparin in the equivalent dosage (22). Ricketts, as well as others, have performed extensive clinical trials with DS and concluded it to be a suitable synthetic substitute for heparin (19, 23, 24).

As tentative hypothesis, then, we believe that dextran sulfate attaches itself to the CFUc cell membrane, thereby resulting in a decreased deformability and increased cell surface negativity. These changes give rise to a mobilization (apparently passive) of CFUc from extravascular sites into the circulation, and possibly also to a transient inability of these cells to leave the blood stream and to "home". This means that the observed increase in CFUc concentration in blood 3–5 hr after DS probably arises from both an elevated influx and a prolonged blood transit time of CFUc.

Summary. Intravenous administration of 10 mg or 15 mg dextran sulfate per kg body weight into dogs results in a marked increase of CFUc in the blood within 3 hr and, to a considerably lesser extent, of mononuclear leukocytes (MNC). The increase after the higher dose is more than tenfold, from less than 200 CFUc/ml blood to about 2000 CFUc/ml, within 3–5 hr; the CFUc gradually decrease thereafter to control levels several hours later. On the other hand, the MNC population experiences a two- to threefold increase which is maintained for several hours. The increase in CFUc after 10 mg/kg is less than after 15 mg/kg, indicating dose-dependence.

There is also a cumulative increase observed in the CFUc concentration in blood after repeated injections of dextran sulfate; there is no significant alteration in PMN and MNC levels and there is a slight decrease in the hematocrit and in the thrombocyte level.

The mechanism responsible for this phenomenon has not yet been clarified. Due to the probable action of polyanions on cell membranes affecting their adherence capabilities, one may consider both an increased rate of release of CFUc from a marginal or extravascular pool into the blood, as well as a prolongation of the CFUc blood transit time as being contributing factors to the observed blood CFUc increase.

We would like to express our sincere thanks to Mrs. B. Burr-Stengel and Miss E. Rüber for their excellent technical assistance, to Dr. I. Steinbach for her splendid animal care and particularly to Dr. H.-P. Schnappauf for his superb surgical skills. Dr. K. J. van den Berg of the Radiobiological Institute, TNO.

Rijswijk, Holland was kind enough to prepare the dextran sulfate.

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Alterations in Bile Flow and Na^+-K^+ Biliary Excretion Induced by Theophylline and Ethacrynic Acid (40043)

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Hepatic canalicular bile production is currently held to be generated by two mechanisms, a bile salt-dependent (BSDF) and bile salt-independent (BSIF) process (1-3). Bile salts are actively transported into the bile canaliculi and subsequent development of osmotic gradients and passive movement of water into the canalicular lumen has been proposed as the means by which bile salts generate bile flow (4). There is more speculation as to the mechanism by which BSIF is generated. Some investigators have implicated cyclic 3'-5'-adenosine monophosphate (cAMP). Theophylline, a phosphodiesterase inhibitor which decreases breakdown of cAMP, has been shown to increase BSIF in the dog, an animal in which very low rates of BSIF have been found (5-7). Observations of the effects of theophylline on BSIF in animal models with high rates of BSIF such as rodents have not been reported. Other investigators have suggested that inhibition of membrane-bound Na^+-K^+-activated adenosine triphosphatase (Na^+-K^+-ATPase) is a factor in BSIF production (8-10). Ethacrynic acid, a diuretic which has been shown to inhibit Na^+-K^+-ATPase in the kidney (11), has been reported to both inhibit (10) and stimulate (12, 13) bile flow without significant change in bile salt output. These studies have been interpreted to support a role for Na^+-K^+-ATPase in the generation of BSIF. In a search for agents which would increase *in vivo* BSIF in the rat, both theophylline and ethacrynic acid were studied. Results of these studies showed that both agents significantly enhanced BSIF. However, both agents also produced alterations in bile salt-dependent flow which suggest they influence multiple steps in the bile secretory process.

Materials and methods. Male Walter Reed rats weighing 250-350 g were used in all experiments. In conducting the research described in this report, the investigator adhered to the Guide for Laboratory Animal Facilities and Care as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council. During ether anesthesia, external biliary fistulae were constructed and cannulation of both the femoral artery and vein was accomplished with PE-10 polyethylene tubing (Clay Adams, Inc.). Bilateral nephrectomy was performed. Animals were then allowed to recover in restraining cages and had free access to food and water for the remainder of the experiments. Studies were performed on the day following surgery after overnight depletion of the bile salt pool. During experiments, body temperature was monitored and maintained at 37 ± 1° with heating pads (14), and animals were perfused with 0.45 ml/hr normal saline. [^{14}C]-Erythritol (Amersham-Searle) was diluted to 3 μCi/ml with sterile normal saline. Grade A sodium taurocholate (Calbiochem) solutions containing 5, 15 and 30 mg/0.45 ml normal saline were prepared on the day of experiment and used for bile salt perfusion. Theophylline and ethacrynic acid were obtained from Vitarine, Inc.

Three microcuries of [^{14}C]erythritol were given through the femoral vein. After a 90 min equilibration period, bile samples were collected at 15 min intervals for 1 hr during the bile salt-depleted state with serum samples obtained midway through the individual bile collection periods via the femoral artery. Each animal then received infusions of taurocholate at 5, 15 and 30 mg/hr; the order of infusion was varied throughout the experiments and did not differ significantly among the three groups. One hour was allowed for equilibration of serum bile salt levels and bile flow between each different

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f bile salt infusion. After each equili-
n period, bile samples were again col-
at 15 min intervals for 1 hr and
obtained midway through each col-
n period. Experimental animals treated
heophylline received 20 mg/kg intra-
sly 15 min before the start of bile
tion. Ethacrynic acid-treated animals
ed 10 mg/kg intravenously 15 min
to the start of bile collection and again
ay through the 1 hr collection period.
ol animals received a comparable vol-
f normal saline. The amounts of drug
in these experiments were selected
monitoring maximum change in bile
and duration of effect produced by
drug in doses from 10 to 40 mg/kg.
lowing each experiment, animals were
ced and liver wet weight determined
netrically. Bile volume was deter-
l gravimetrically. Bile salt concentra-
were measured using the hydroxyste-
lehydrogenase method as modified by
rand and Small (15). Sodium and po-
m concentrations in bile were mea-
by an IL Flame Photometer, Model
(Instrumentation Laboratory, Inc.).
activity in bile and plasma was deter-
l in a Beckman LS-345 scintillation
n using the external standard ratios to
t for quenching. Biliary clearance of
rythritol was calculated as the prod-
f bile flow and the ratio of bile/plasma
adioactivity. Correlations between bil-
[^{14}C]erythritol clearance versus bile
nd bile salt output and bile flow versus
m and potassium excretion were cal-
d for individual animal experiments
rol n = 5, Ethacrynic acid n = 7,
phylline n = 5], using the method of
squares (16). The y-intercept of the
sion line calculated for the relation-
f erythritol clearance to bile acid out-
used to describe BSIF while the slope
regression line for this relationship
sents the amount of choleresis/unit of
cid (BSDF). Similar terminology has
used in the interpretation of the
sion lines calculated for bile flow ver-
liary Na^+ and K^+ output. The unpaired
nt t test was used to compare differ-
in mean slopes and y-intercepts be-
two animal groups in which linear regression lines were calculated from data attained from each individual animal in the group. Analysis of covariance was also used to compare differences in the composite regression lines obtained for these comparisons between the control group of animals and the two drug-treated groups (16).

Results. Erythritol clearance showed a linear relationship to total bile flow in control animals and in both the theophylline (TH) and ethacrynic acid-treated (EA) groups (Fig. 1). Bile/plasma [^{14}C]ratios approximated unity and were not significantly different among the three groups (Control 1.006 ± 0.040, TH 0.960 ± 0.084, EA 0.973 ± 0.048). These data suggest that bile flow was originating from the hepatocyte canaliculus in all three experimental groups and that there was little alteration of canalicular bile by ductular secretion or reabsorption. However, TH and EA both altered the relationship between erythritol clearance and bile salt output as compared to controls (Fig. 2 and Table I). Both agents significantly increased the fraction of erythritol clearance which was independent of bile salt output ($P < 0.001$). In addition, theophylline produced a significant decrease in the erythritol clearance generated per unit of bile salt secreted as compared to the

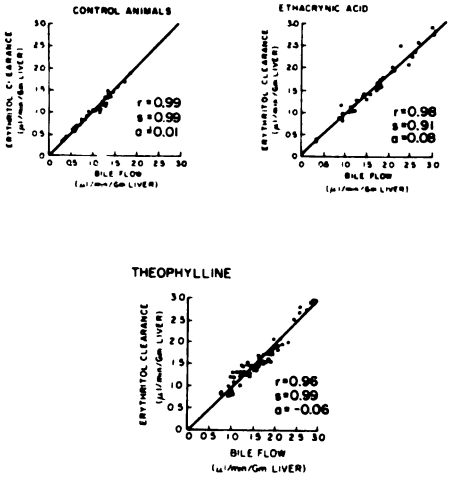

FIG. 1. Relationship of erythritol clearance to bile flow in control and both experimental groups is linear with a slope which approximates unity. No significant difference exists for this relationship between control and either experimental group.

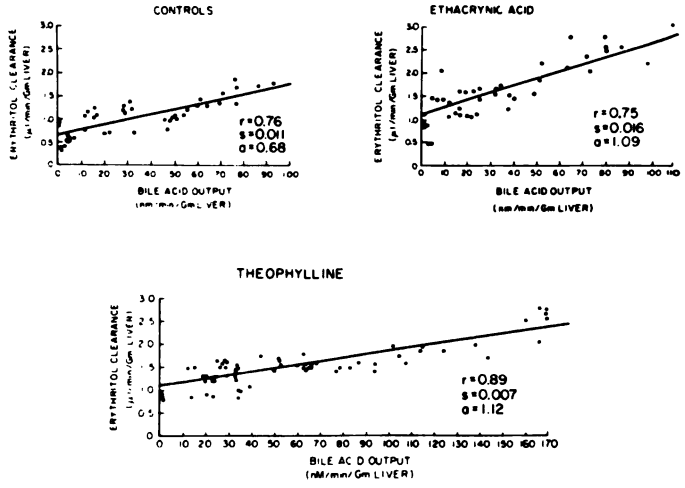

FIG. 2. Linear regression lines showing the relationship of erythritol clearance to bile acid output in control and experimental groups. Bile salt-independent flow (y-intercept) is significantly increased by both EA and TH treatment ($P < 0.001$). The bile flow generated per unit bile acid secretion (slope) is significantly increased by EA ($P < 0.025$) and significantly decreased by TH treatment ($P < 0.001$).

TABLE 1. MEAN VALUES FOR SLOPES AND Y-INTERCEPTS OF REGRESSION LINES CALCULATED FROM COMPARISON OF ERYTHRITOL CLEARANCE VERSUS BILE ACID OUTPUT AND BILE FLOW VERSUS BILIARY NA^+ AND K^+ EXCRETION IN CONTROL, ETHACRYNIC ACID AND THEOPHYLLINE TREATMENT GROUPS.[a]

	Erythritol clearance versus bile acid output[b]		Bile flow versus biliary Na^+ excretion		Bile flow versus biliary K^+ excretion	
	Slope	Y-intercept	Slope	Y-intercept	Slope	Y-intercept
Control ($n = 5$)[c]	0.0120 ± 0.0011	0.585 ± 0.217	0.5385 ± 0.0749	0.1297 ± 0.0968	0.8685 ± 0.1352	0.1667 ± 0.0884
EA ($n = 7$)	0.0145 ± 0.0016 ($P < 0.025$)	1.254 ± 0.159 ($P < 0.001$)	0.5583 ± 0.0497	0.1587 ± 0.1087	1.0183 ± 0.1010	0.3535 ± 0.1291 ($P < 0.05$)
TH ($n = 5$)	0.0062 ± 0.0007 ($P < 0.001$)	1.148 ± 0.027 ($P < 0.001$)	0.5006 ± 0.0543	0.3120 ± 0.1130 ($P < 0.05$)	1.2129 ± 0.2215 ($P < 0.05$)	0.4116 ± 0.1815 ($P < 0.05$)

[a] P values listed in parentheses below mean when significantly different from control group.
[b] Mean ± SD.
[c] Number of animals in group.

control experiments ($P < 0.001$). Bile acid outputs for the theophylline experiments were also somewhat higher than those seen during similar bile salt perfusion rates in the control and EA treatment groups (see Fig. 2). It is possible that TH has some effect on the transport of bile acids such that given levels of serum and hepatic bile acids result in higher biliary bile acid outputs after TH in comparison to control or EA treated animals. Recalculation of the linear regression line for erythritol clearance versus bile acid output in the TH group using only values for bile acid output similar to those seen in the control and EA groups (<100 nM/min/Gm liver) resulted in a slope and y-intercept which differed insignificantly from those obtained using all the TH data. Ethacrynic acid produced the opposite effect of theophylline with more erythritol cleared per unit bile salt secretion than was seen in controls ($P < 0.025$). Separate linear regression lines for erythritol clearance versus bile acid output during the different rates of bile salt perfusion were calculated for the ethacrynic acid and theophylline groups. This was done to insure that the changes in erythritol clearance per unit bile salt secretion were not due to variations in degree of BSIF alteration by EA and TH during different rates of bile salt perfusion. The calculated bile salt-independent choleresis induced by both EA and TH was similar for each rate of bile salt

perfusion, indicating that differences in the amount of bile generated per unit bile salt secretion produced by these agents is not due to changes in magnitude of BSIF at varying rates of bile salt perfusion.

Since changes in active transport of Na^+ and K^+ have been proposed as a mechanism by which BSIF is generated, Na^+ and K^+ biliary excretion were plotted against bile flow in the three experimental groups (Figs. 3, 4, Table I). Bile flow was linearly related to total Na^+ biliary excretion in the controls and in both the TH and EA animals. There was no significant difference in the amount of bile flow generated per unit of Na^+ excretion among the three treatment groups. Little bile flow was independent of biliary Na^+ excretion in the control group. However, an increased amount of bile flow appeared to be independent of biliary Na^+ excretion in both the TH and EA groups, and this increased Na^+ independent bile flow in the theophylline group differed significantly from that seen in the control group ($P < 0.05$). Both TH and EA produced similar changes from the control group in relationship of bile flow to biliary K^+ excretion. Significantly more bile was secreted per unit of biliary K^+ excreted in the theophylline group as compared to the control group ($P < 0.05$), and this comparison approached significance in the ethacrynic acid-treated group ($P > 0.05$). Both agents produced a significant increase in K^+-independent bile flow ($P < 0.05$).

Discussion. In this study both theophylline, a phosphodiesterase inhibitor which would be expected to elevate intracellular

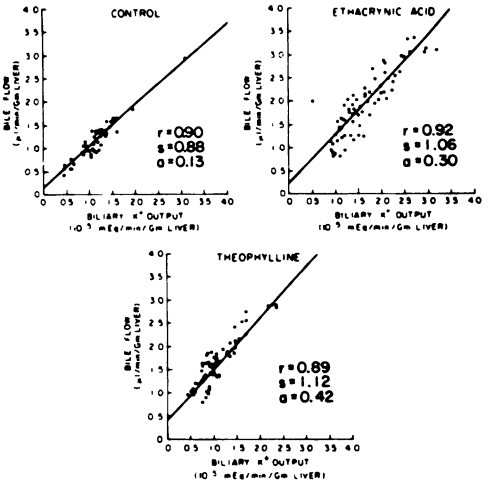

FIG. 4. Linear regression equations for bile flow versus biliary K^+ excretion show that the bile flow per unit K^+ excretion (slope) is increased by both EA ($P > 0.05$) and TH ($P < 0.05$). Both agents also produced an increase in bile flow independent of biliary K^+ excretion ($P < 0.05$).

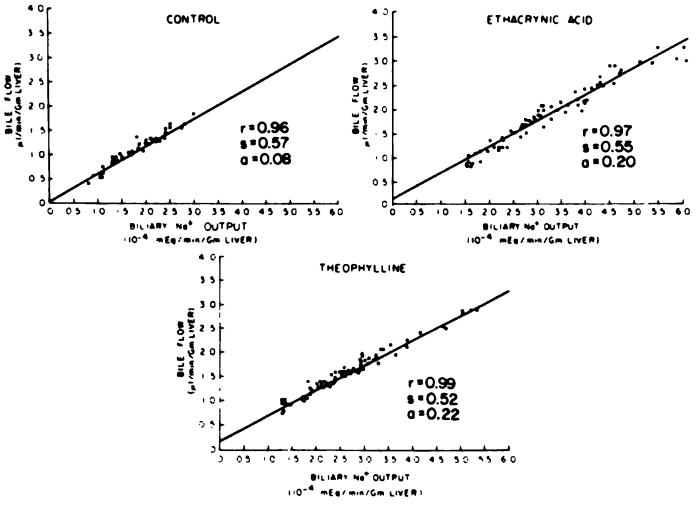

FIG. 3. Comparison of the relationship of bile flow to biliary Na^+ excretion shows no significant differences among the control and experimental groups for the volume of bile flow generated per unit Na^+ excreted. Bile flow independent of biliary Na^+ excretion (y-intercept) was increased in both the EA and TH groups, and this increase was statistically significant for TH ($P < 0.05$).

levels of cAMP, and ethacrynic acid, a Na^+-K^+-ATPase inhibitor, produced approximately equivalent increases in *in vivo* BSIF in the rat. While biliary excretion of TH and EA was not measured in this study, it is unlikely that the osmotic effect of either drug or its metabolites accounts for these increases in BSIF and alterations in biliary electrolyte excretion. Measurement of biliary TH in dogs indicates that amounts excreted into bile are insufficient via their osmotic effects to account for the increase in bile flow produced by this agent (5, 6). A recent report suggested an osmotic mechanism for EA-induced choleresis in the rat (17), but a comprehensive study of hepatic metabolism and biliary excretion of EA in the rat showed asynchronism between the choleretic effect of EA and its appearance in bile (18).

Proposed mechanisms involved in the generation of BSIF have centered primarily around cAMP and Na^+-K^+-ATPase. Proponents of a role for cAMP point to the increased BSIF induced by TH in dogs (5, 6) and increased bile flow in both dogs and humans in response to glucagon which enhances intracellular concentration of cAMP (19, 20). Since active Na^+ transport in other cell membranes is associated with changes in Na^+-K^+-ATPase and this enzyme has been isolated from rat liver homogenates (21, 22), other investigators favor alterations in Na^+-K^+-ATPase as the mechanism responsible for changes in BSIF (8–10). Both TH and EA increased BSIF in this study and both agents produced similar changes in the relationship of bile flow to biliary K^+ excretion which included an increase in bile flow per unit K^+ excretion and an increase in bile flow independent of K^+ excretion. This suggests that at least some part of the increased BSIF produced by both agents is due to alterations in electrolyte transport. It is conceivable that TH induces changes in Na^+-K^+-ATPase to account for its stimulatory effect of BSIF or that EA produces alterations in intracellular cAMP levels. It remains to test these drugs in this regard. However, it is quite likely that production of BSIF is dependent on multiple mechanisms and that both the cAMP and Na^+-K^+-ATPase systems are important in the generation of bile flow independent of bile salt secretion.

In addition to its effect on BSIF, TH also altered bile salt-dependent bile flow by decreasing the volume of bile secreted per unit of bile salt secretion. Several possible mechanisms could explain this change. Theophylline can produce vasoconstriction with resultant reduction in hepatic plasma flow. While generation of BSIF appears to be independent of wide fluctuations in hepatic plasma flow (23), the effects of decreased hepatic plasma flow on generation of BSDF have not been carefully examined. Theophylline-induced alteration of bile salt-cation interrelationships might also explain the decreased bile flow generated per unit bile salt secretion. It has been shown that activity coefficients for Na^+ and K^+ in bile salt solutions are less than the corresponding values one could expect in NaCl and KCl solutions (24). Loss of osmotic activity resulting from changes in bile salt-cation interaction produced by TH could account for the decreased bile flow generated per unit bile salt secretion. In contrast to theophylline, ethacrynic acid produced an opposite effect with increased bile flow per unit bile salt secretion when compared to controls. In a study of the effects of EA on bile flow in multiple animal species, Shaw also found an increased bile flow per unit bile salt output (13). However, no erythritol clearances were performed in this study and the data was not subjected to statistical analysis. These data suggest that the mechanism for generation of BSDF may not be as simplistic as has been previously postulated.

Summary. Both theophylline, a phosphodiesterase inhibitor, and ethacrynic acid, a Na^+-K^+-ATPase inhibitor, produced similar increases in BSIF in the rat. Whereas theophylline significantly decreased BSDF, ethacrynic acid increased BSDF. These results suggest that the mechanism responsible for generation of both BSIF and BSDF are multifactorial and further study will be necessary to define them.

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An *in Vitro* Transformation Assay for SiSV-1 and HL23V Using Feline Embry Fibroblasts (40044)

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Simian sarcoma virus (SiSV-1) was first observed in and isolated from a fibrosarcoma of a woolly monkey (1-3), and has been shown to produce fibrosarcomas in newborn marmosets (2). Furthermore, several culture lines, including marmoset, human, cat, and rat cells were reported to be susceptible to transformation by this virus (3-5).

HL23V was isolated from the cultured peripheral blood and bone marrow leukocytes of a patient with acute myelogenous leukemia and was shown to be infectious for a wide variety of cell lines (6-8). An isolate of HL23V has the ability to induce tumors in marmosets and transform cultured marmoset cells (9) and has been shown to be a mixture of viruses related to both SiSV-1 and baboon endogenous virus (10-12). Two other primate type-C viruses used in this study, baboon endogenous viruses (BaEV) (13-15) and gibbon ape leukemia virus (GaLV) (16-18) have been shown to be nontransforming for a number of cultured cell lines.

In this report the focus-formation titer of SiSV-1 and HL23V on feline embryo fibroblast cells is compared to that using other commonly used cell lines, including; normal rat kidney; rat embryonic fibroblast; and several cultures of primate origin. Transformation by these viruses was found to be substantially more rapid and sensitive when FEF cells are used. A similar observation was recently reported by Sarma and Law (19).

Materials and methods. Cells and v
The various cell cultures and viruse in this study are described in Table cells were grown in McCoy's 5A m media (Gibco, Grand Island, NY) s mented with 10% heat inactivate bovine serum (Reheis Chemical Cor Phoenix, Arizona) and carried in 3024 culture flasks at 37° and 5% C humid atmosphere. Cultures were t ized and passaged every 4-5 days as using a 0.25% trypsin-EDTA s (Gibco, NY). Virus and cell stock stored in liquid nitrogen.

Transformation assay. Cells to be i were seeded at 5×10^5 per 60 mm dish (Falcon 8083) or 7×10^4 per w 24 well Linbro Plate (Linbro Sci Inc., Hamden, CO). Cultures wer treated as follows: at 5-18 hr postse fluid was replaced with 25 μg/ml D Dextran (Pharmacia, lot #273 McCoy's 5A unsupplemented medi cubated at 37° for 30 min, and then twice with phosphate buffered saline Int. Biological Lab., Inc., Rockville. Serial tenfold dilutions of virus or media (0.5 ml per 60 mm dish or per Linbro well) were added to du cultures for 1 hr at 37°. After virus tion, complete medium was added a tures incubated. Twenty-four hour infection, NRK-infected cells were c to medium containing 5% serum, an cells were refed with and maintai regular growth medium. All culture carefully monitored to maintain a pH. Foci were counted at the fo times after infection of the indicat lines: feline embryonic cells, 3-4 days cells, 7-8 days; and all other cell li 10 days. After optimum focus develo all monolayers were rinsed with stained for 5 min with a mixture of

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TABLE I. CELLS AND VIRUSES.

Cells	Designation	Source
Bat lung	CCL88	ATCC, Rockville, MD.
Fetal canine thymus	Cf2Th[a]	Naval Biological Laboratories, Oakland, CA.
Feline embryo fibroblast	FEF-1	Dr. P. Sarma, NIH, Bethesda, MD. (#D843)
Feline embryo fibroblast	FEF-2	Dr. S. Ruscetti, NIH, Bethesda, MD.
Feline embryo fibroblast	FEF-3	Dr. J. Whitman, HEM Research, Rockville, MD. (FEE-#6)
Feline embryo fibroblast	FEF-4	Naval Biological Laboratories, Oakland, CA. (FC113)
Feline embryonic tongue fibroblast	CCL176	ATCC, Rockville, MD.
Human embryo fibroblast	PTA-11	Dr. R. C. Gallo, Lab. of Tumor Cell Biol., NCI, Bethesda, MD.
Human embryo fibroblast	WHE-2	Dr. R. C. Gallo, Lab. of Tumor Cell Biol., NCI, Bethesda, MD.
Human foreskin fibroblast	HFF	Drs. F. Deinhardt and C. Bergholz, Rush-Presbyterian-St. Lukes Med. Center, Chicago, IL.
Marmoset skin fibroblast	HF	Drs. F. Deinhardt and C. Bergholz, Rush-Presbyterian-St. Lukes Med. Center, Chicago, IL.
Marmoset skin fibroblast	MFS	Drs. F. Deinhardt and C. Bergholz, Rush-Presbyterian-St. Lukes Med. Center, Chicago, IL.
Marmoset lung fibroblast	1283	Drs. F. Deinhardt and C. Bergholz, Rush-Presbyterian-St. Lukes Med. Center, Chicago, IL.
Rat embryo fibroblast (Fisher)	RE-1	Dr. R. Ting, Biotech, Rockville, MD. (#112)
Rat embryo fibroblast (Fisher)	RE-2	Dr. R. Ting, Biotech, Rockville, MD. (#810)
Normal rat kidney	NRK-1[b]	Dr. R. Ting, Biotech, Rockville, MD. (Clone #153-S)
Normal rat kidney	NRK-2[b]	Dr. R. Ting, Biotech, Rockville, MD. (Clone #13)
Normal rat kidney	NRK-3[b]	Dr. R. Ting, Biotech, Rockville, MD.
Normal rat kidney	NRK-4[b]	Dr. R. Bassin, NIH, Bethesda, MD. (#D173HC)

Viruses	Designation	Source
Baboon endogenous virus (M7)	BaEV[c]	Dr. G. Todaro, NIH, Bethesda, MD.
Gibbon ape leukemia virus	$GaLV_H$[d]	Dr. R. C. Gallo, Lab. of Tumor Cell Biol., NCI, Bethesda, MD.
Human leukemia virus (HL23V)	HL23V[e]	Drs. R. Gallagher and R. Gallo, NIH, Bethesda, MD.
Simian sarcoma virus	SiSV-1[f]	Drs. F. Deinhardt and C. Bergholz, Rush-Presbyterian-St. Lukes Med Center, Chicago, IL.

[a] Normal fetal canine thymus (Cf2Th) described by Nelson-Rees *et al.* (20).

[b] Normal rat kidney cells described by Duc-Nguyen *et al.* (21).

[c] Baboon endogenous virus (14) maintained in chronically infected dog thymus cells (20).

[d] Virus isolate ($GaLV_H$) (Gallo, R. C., Gallagher, R. E., Wong-Staal, F., Aoki, T., Markham, P. D., Schetters, H., Ruscetti, F., Valerio, M., Saxinger, W. C., Smith, R. G., Gillespie, D. H., and Reitz, M. S., Virology, in press, Feb. 1978), passed to and produced in bat lung cells (CCL88).

[e] HL23VI-1p10 (6-8) isolated and passed in dog thymus cells.

[f] SiSV (2) grown in marmoset cells and passed to and produced in bat lung cells (CCL88).

(Harelco, Phila., PA) and methanol (absolute) and rinsed with tap water. The focus forming titer of each virus was calculated from the average number of foci on cultures infected with the serially diluted virus.

Transformation assay under agar. Assays were done in cluster dishes (Falcon 3004, 35 mm × 4 wells per dish) in quadruplicate. Cells were seeded (3×10^5 per well) and allowed to incubate for approximately 18 h. Cultures were then infected and allowed to incubate for an additional 20 hr at 37° and 5% CO_2. At this time culture fluid was removed and the culture overlayed with 2.5 ml of 0.3% agar (Difco Lab., Detroit) dissolved in McCoy's 5A media supplemented with 15% fetal bovine serum. The cultures were carefully monitored for optimum focus formation for approximately 8 days.

Serum neutralization. Immune serum and virus were diluted in unsupplemented McCoy's 5A medium, mixed in equal

amounts, and incubated for 4 hr at 4° prior to adsorption to monolayers according to procedures described above. Goat anti-SiSV-1 serum raised against virus infected cells was obtained from Dr. C. Bergholz (Rush-Presbyterian-St. Lukes Medical Center, Chicago, IL.).

Viral interference assay. Cultures were infected as described above with sequential one to ten dilutions of the virus stock extending beyond the transforming endpoint of the virus. Infected cultures were subcultured at confluency three times and challenged using procedures described above with a known titer of transforming virus. A 90% reduction in the number of foci produced on the challenged cultures compared to that on control cultures was interpreted as being positive for interference.

Viral DNA polymerase assay. Culture fluids were monitored for viral DNA polymerase activity, using $(dT)_{15-18} \cdot (A)_n$ and $(dT)_{15-18} \cdot (dA)_n$ as primer-templates (7).

Results. Several cell lines were compared in their sensitivity to transformation by several primate type-C viruses. The results of one experiment are shown in Table II and are representative of data obtained in a number of experiments using the cell lines shown, as well as with several other human embryonic cell lines. Conditions needed for each virus and cell line to yield optimum transformation had been previously determined. The NRK-1 and RE-1 cell lines used in this study had been preselected for their sensitivity to transformation by SiSV-1 (R. Ting, personal communication). As seen in Table II, SiSV-1 or HL23V were able to induce foci in all of the cell lines tested, whereas $GaLV_H$ and BaEV gave no indication of transforming any of the tested cell lines as expected from previous reports (13–18). The viruses SiSV-1, HL23V, and $GaLV_H$ were able to replicate in all cell lines shown, whereas BaEV gave no indication of replicating in the NRK, RE, or FEF cells. This information was obtained by continued subculturing of infected cultures through at least five passages and assaying for the release of virus by testing for extracellular viral DNA polymerase activity.

TABLE II. COMPARISON OF THE TRANSFORMING TITER OF FOUR PRIMATE RNA TUMOR VIRUSES ON SEVERAL CELL LINES.

	Virus[a]			
Cell[a] (b)	SiSV-1 $\times 10^4$ ffu/ml	HL23V $\times 10^4$ ffu/ml	$GaLV_H$ ffu/ml	BaEV ffu/ml
FEF-1 (37)	11	4.2	0	0
NRK-1 (14)	5	1.5	0	0
RE-1 (3)	7	3.9	0	NT
PTA-11 (9)	0.1	ND	0	0
WHE-2 (14)	0.1	ND	ND	0
HFF (22)	1.2	0.9	0	0
HF (29)	0.1	0.02	0	0
1283 (22)	0.1	0.04	0	0
MFS (15)	ND	0.003	ND	ND

[a] Cells and viruses are described in Table I.
[b] Passage level.

In these preliminary studies, two observations were noteworthy. One was the difference in transforming titers of virus stocks when compared on the different cell lines. The other was the comparative rapidity of the assay when FEF-1 cells were used. In this regard, foci were completely developed and ready for counting by the third or fourth day after infection of FEF-1 cells, whereas all other cell lines required from 7 to 10 days to give maximum focus development.

To further test the apparently higher sensitivity of the FEF-1 cells to transformation by SiSV-1 and HL23V, a number of experiments were conducted comparing selected cell lines using standard stocks of virus. In Table III the transforming titer of virus on each cell line tested is expressed as a percentage of the titer obtained on FEF-1 cells used as a control in each experiment. Each percentage recorded represents an independent experiment. For example, in four independent experiments, SiSV-1 gave titers on NRK-1 cells 24, 50, 55, and 10% of those obtained using FEF-1 cells. Experiments were considered valid only when viral stocks gave the expected titer on the FEF-1 control cells, i.e., 10^4–10^5 ffu/ml for SiSV-1 and 10^3–10^4 ffu/ml for HL23V.

As mentioned above, the NRK-1 and RE-1 cultures were specifically selected because of their sensitivity to transformation by SiSV-1. This is apparent from the results shown in Table III where a number of NRK cultures and one other RE cell line was used.

To determine if the sensitivity to transfor-

3LE III. SENSITIVITY OF SELECTED CELL TURES TO TRANSFORMATION BY SiSV AND HL23V RELATIVE TO FEF-1.[a]

	Virus[b]	
ll[b] (c)	SiSV-1 (%)	HL23V (%)
0–45)	100	100
–5)[d]	68;63	74;75
)	3	6
–6)	ND	55;81
(17–20)	10;8	ND
13–15)	24;50;55;10	17;33;33;38;38
25–27)	0.5;0.3	16;6
3–5)[d]	0.7;0.8	12;15
137–142)	0.8;0.8	10;13;16;18
5)	62;45;65	150;47;93
6)	4	10;14
(9–12)	1.0;2.0;1.1	<1.0;<1.0
12–21)	1.1;1.0;1.0	<1.0;<1.0
–30)	11;25;11	22;42;39;19
31)	3.6;1.2;1.1	0.5;0.7
–25)	1.1;5.4;1.2	1.1;0.7
–17)	ND	0.1;0.7

focus forming titer of each virus is expressed ent of that obtained using FEF-1 cells as the control in each experiment. Each percentage s an independent experiment.

s and viruses described in Table I.

range of passage levels used during the course studies.

passage level history is not known. Cultures saged the indicated number of times in our y.

Not done.

observed for FEF-1 was generally feline cells, other feline cell lines so included in our comparative transon studies (shown in Table III). Of feline embryonic cell lines tested, d a sensitivity to transformation by and HL23V comparable to FEF-1. comparative transforming titer of and HL23V on three human embryll lines and three cell cultures of set origin is also illustrated in Table th the exception of the human foreproblast (HFF) cells these cultures pproximately 100-fold less sensitive EF-1. The sensitivity of the HFF cell ransformation by SiSV-1 and HL23V antially greater than several other cell lines that have been tested and ven to be very useful in the study of ruses.

ew of the apparent higher sensitivity cells to transformation by SiSV-1 L23V compared to other cell lines tested, it was important to rule out the possibility that the difference was due to nonspecific or secondary focus development. In this regard, the FEF-1 cells did not give any indication of spontaneous focus development under the conditions of our assay. Not only were numerous mock infected controls consistently negative, but also cultures treated with non-transforming viruses gave no indication of focus development (Table III). Furthermore, SiSV-1 induced focus development was completely neutralized by antiserum prepared in goats against SiSV-1 (Table IV). The virus produced by SiSV-1 transformed FEF cells retains its ability to induce foci on normal FEF cell lines (data not shown), however, it is unlikely that the development of secondary foci is responsible for the increased number of foci seen since under the conditions of our assay on FEF the optimum focus development coincides with the cells reaching confluency and both occurred very rapidly, i.e., by the third or fourth day after infection. Also, as shown in Table V, under the conditions of the focus assay used, it was not necessary to use an agar overlay to

TABLE IV. NEUTRALIZATION OF SiSV-1 INDUCED FOCI ON FEF-1 BY ANTI-SiSV-1 SERUM.[a]

Serum		Number foci[b]	% Reduction
None		77	0
Immune serum	1:10	0	100
dilution	1:100	0	100
	1:200	4	95
	1:300	54	30

[a] Viral stocks were preincubated with or without dilution of immune serum and assayed for transforming activity as described in *Materials and methods*.

[b] Average of duplicate cultures.

TABLE V. TRANSFORMATION OF FEF-1 CELLS WITH OR WITHOUT AGAR OVERLAY.

Virus[a]					
SiSV-1 × 10^4 ffu/ml[b]			HL23V × 10^2 ffu/ml[b]		
Agar		*No Agar*	*Agar*		*No Agar*
Day 3	*Day 8*	*Day 3*	*Day 3*	*Day 8*	*Day 3*
3.50	3.75	3.12	1.26	1.50	1.98

[a] Virus and cells are described in Table I.

[b] The virus titer was calculated from the average of quadruplicate dishes counted on the indicated number of days post infection.

avoid secondary or satellite focus development.

Discussion. A number of cell lines were compared for efficiency of transformation by SiSV-1 and HL23V. The assay was consistently more rapid and sensitive with feline embryonic cells than with rat (NRK and RE) or several human and marmoset cell lines. The results obtained with FEF-1 cells are apparently not unique to one specific feline embryonic cell line since two other cultures gave comparable results. Feline embryonic cells have been previously reported to be four times more sensitive than marmoset cells to transformation by feline sarcoma virus (22).

The basis for the enhanced sensitivity of feline embryonic cells to transformation by SiSV-1 and HL23V is not known, but it is likely related to relative differences in susceptibility to infection by the viruses, rather than a difference in expression of viral transforming information. This is implied by the data in Table VI, which demonstrates that the titers of both the transforming and nontransforming components of SiSV-1 are shifted proportionally downward on human embryonic cells (PTA-11) compared to FEF-1 cells.

The mechanisms responsible for the observation that feline and rat cells are more sensitive than are the other cells studied to infection and/or transformation by some type-C viruses are not known, but a contributing factor could be that they both contain inducible endogenous viral information (23).

Both SiSV-1 and HL23V behaved in a similar manner in transformation experiments with respect to time needed for maximum focus development and morphology of the induced foci. The foci induced by HL23V or SiSV-1 were densely packed, stellate arranged, and fusiform (Fig. 1). On the other hand, foci induced on feline embryonic cells by feline sarcoma virus, are reported to have a distinctly different morphology (less dense, flat, round, or fusiform [22]). Another similarity between the viruses (Table VI) is that the non-transforming components of both SiSV-1 and HL23V are present at an approximately two logs higher titer than the transforming component. The same is also true for SiSV-1 tested on PTA-11 cells. A possible difference between the two viruses (SiSV-1 or HL23V) was, however, seen in their transformation behavior on rat cells compared to that on FEF cells (Table III). With the exception of the NRK-1 cultures, HL23V was consistently more efficient in its ability to transform the rat cell lines relative to FEF-1 than was SiSV-1.

In conclusion, transformation assays using FEF cells offers several advantages over other commonly used cell lines. The rapidity of focus development, i.e., 3–4 days versus 7–10 days, could facilitate progress in experiments involving the use of large numbers of focus assays. Also, the increased sensitivity of the cells to infection by type-C viruses could be critical when low levels of viral activity may be present. Finally, FEF cells have also been found to be target for a mixed cell syncytial (24, 25) assay with XC cells (our personal observations). This, therefore, permits the assay and study

TABLE VI. TITER OF TRANSFORMING AND NONTRANSFORMING ACTIVITIES OF SiSV-1 AND HL23V.[a]

		Initial SiSV-1 dilution							Initial HL23V dilution				
Cell[b] (c)	Activity	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
FEF-1 (35)	Transformation[d]	+	+	+	+	0	0	0	+	+	+	0	0
	Interference[e]					+	+	0				+	+
PTA-11 (10)	Transformation[d]	+	+	0	0	0							
	Interference[e]			+	+	0							

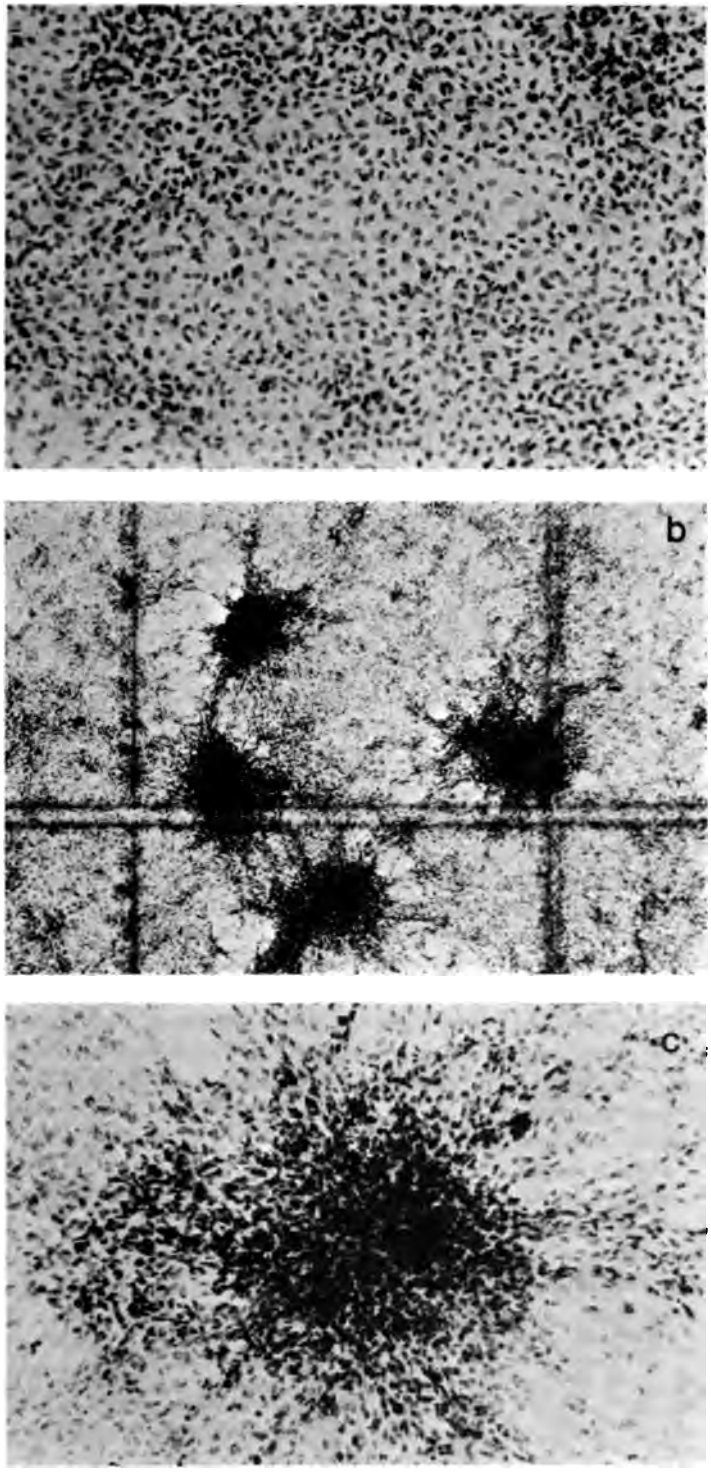
[a] Serial tenfold dilutions of each virus was used to infect cell cultures. Transformed cultures were noted and all cultures were subcultured three times, before challenging with a known transforming titer of virus.

[b] Cells and viruses are described in Table I.

[c] Passage level.

[d] + indicates cultures containing viral induced foci; 0 indicates no transformation.

[e] + indicates at least 90% reduction of foci in challenged cultures compared to control cultures, i.e., positive interference; 0 indicates transformation comparable to control cultures, i.e., no interference.



1. Normal and HL23V transformed feline embryonic fibroblast cells. FEF-1 were infected with HL23V nd stained with giemsa methanol as described in *Materials and methods*. Uninfected FEF-1 cells, ×410 nsformed FEF-1 cells, ×120 (b), and ×410 magnification (c).

of transforming and nontransforming virus titers on the same culture. Rat cell lines have been used in XC syncytial assay with these and other mammalian viruses (7, 25) but have not been suitable, in our hands, for use with these viruses.

Summary. Feline embryo fibroblast cells were found to be very sensitive to transformation by the primate RNA tumor viruses, simian sarcoma virus (SiSV-1) and HL23V, a virus isolated from the leukemic myeloblasts of an AML patient. *In vitro* transformation of feline embryo fibroblast cell cultures with these viruses was 2 to 100 × more efficient than when performed on the normal rat kidney (NRK) or rat embryo fibroblast cell lines commonly used for SiSV-1 transformation studies. Several cell lines of marmoset and human origin were also observed to be less sensitive to transformation by these viruses. Furthermore focus development on feline embryonic fibroblast cultures required only 3–4 days compared to 7–10 days needed using rat or primate cultures.

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DINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 319-321 (1978)

Sex- and Line-Dependent Differences of Renal α-Galactosidase and β-Galactosidase Activities in Two Highly Inbred Lines of Chinese Hamsters with Spontaneous Diabetes (40045)

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epression in the activities of several l glycohydrolases has been reported in streptozotocin-diabetic rats and postul to be a contributory factor leading to oangiopathy (1). Thickening of capilbasement membrane is widely regarded ıe basis of diabetic microangiopathy and related difference has been observed in width of muscle capillary basement ıbrane in humans (2). This investigation efore was performed to determine if dependent variation in the levels of l glycohydrolases also exists in two ly inbred lines of Chinese hamsters, with spontaneous glycosuria and hyperemia.

aterials and methods. The animals were cted from a Chinese hamster colony ıtained for the production of genetir diabetic animals (3). The M line has luced only aglycosuric offspring in the ten generations and the XA line con-ed over 90% glycosuric animals. The ıals of the XA line used in this study, ever, were all glycosuric.

fter exsanguination through orbital s, the kidneys were removed and ho-genized in 9 vol of chilled buffer contain-0.45 *M* sucrose, 0.68 m*M* EDTA ad-ed to pH 7.0 with 0.5 *M* NaOH in er-Elvehjem vessel. The homogenates e centrifuged at 12,000*g* for 20 min. pellets were washed once with the same er, suspended in 9 vol (tissue weight) ıuffer, frozen and thawed thrice and ıogenized in a Polytron homogenizer/ ıtegrator at a setting of seven for 15

Nitrophenyl derivatives were used as trates for glycohydrolase assays at a centration of 5.26 m*M* in a buffer made djusting 0.1 *M* Na_2HPO_4 to desired pH 0.05 M citric acid. *N*-Acetyl-β-D-hexninidase, α-galactosidase and β-galactosidase were assayed at pH 4.5, α-glucosidase at 6.5 and β-glucosidase at 5.5. The assays followed the procedure of Felton *et al.* (4) and were run in a four point time course. One unit of enzyme was defined as the amount which hydrolyzed one nmole substrate per min. Blood sugar and protein were determined as described previously (3).

Column chromatography (0.9 × 30 cm) was carried out with Cellex D suspended in 0.01 *M* NaH_2PO_4, pH 6.0 and eluted with a linear gradient made by mixing 100 ml each of 0 and 0.25 *M* NaCl in the same buffer. Four ml kidney supernatant fraction from a M line female animal was chromatograph and 0.1 ml aliquots of 2.2 ml fraction were assayed for α-galactosidase and β-galactosidase activities in even-numbered tubes.

Results and discussion. Table I summarizes all data obtained in this study and they are expressed as means ± SEM of six animals in each group.

The animals were matched according to age and the males were significantly heavier than the females in the same line. The diabetic XA animals also tended to be slightly heavier that the M animals of the same sex. No significant difference, however, was observed in the kidney weight, expressed as % body weight in view of the difference in the body weights, in all four groups of animals. The female M animals showed significantly less soluble proteins in the kidney than the male counterparts and the significance of this observation remains unclear.

In both 12,000*g* supernatant and precipitate fractions, *N*-acetyl-β-D-hexosaminidase was present in the highest concentration and about 40% of its activity was recovered in the supernatant. Both males and females in the same inbred line showed similar activ-

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TABLE I. AGE, BODY AND KIDNEY WEIGHTS, BLOOD SUGAR AND RENAL GLYCOHYDROLASES IN MALE AND FEMALE CHINESE HAMSTERS OF M AND XA LINE.[a]

	M-Line			XA-Line		
	Male	Female	P^b	Male	Female	P^b
Age (weeks)	19.7 ± 3.6	20.0 ± 2.5	NS	20.2 ± 3.4	18.8 ± 2.0	NS
Body weight (g)	27.3 ± 1.5	18.9 ± 0.5	<0.001	29.2 ± 2.3	23.8 ± 1.0	<0.05
Blood sugar (mg/dl)	128 ± 5	125 ± 10	NS	362 ± 50	369 ± 50	NS
Kidney weight (% body weight)	1.00 ± 0.03	1.08 ± 0.03	NS	1.10 ± 0.04	1.13 ± 0.04	NS
12,000g supernatant						
protein concentration (mg/g)	91.7 ± 2.9	82.3 ± 1.7	<0.025	86.7 ± 2.2	88.9 ± 2.8	NS
N-acetyl-β-D-hexosaminidase (U/g)	1723 ± 85	1961 ± 150	NS	1607 ± 188	1898 ± 147	NS
α-glucosidase (U/g)	105 ± 6	97 ± 6	NS	112 ± 6	110 ± 5	NS
α-galactosidase (U/g)	702 ± 68	1176 ± 131	<0.005	392 ± 41	734 ± 82	<0.005
β-glucosidase (U/g)	998 ± 66	899 ± 26	NS	939 ± 54	830 ± 52	NS
β-galactosidase (U/g)	1186 ± 71	1837 ± 97	<0.001	810 ± 53	1304 ± 194	<0.05
12,000g precipitates						
protein concentration (mg/g)	82.8 ± 6.3	82.2 ± 4.0	NS	91.3 ± 5.1	86.6 ± 4.7	NS
N-acetyl-β-D-hexosaminidase (U/g)	2401 ± 221	2527 ± 142	NS	2409 ± 239	2749 ± 189	NS
α-glucosidase (U/g)	40 ±4	35 ± 2	NS	42 ± 3	38 ± 2	NS
α-galactosidase (U/g)	458 ± 43	438 ± 17	NS	385 ± 26	422 ± 26	NS
β-galactosidase (U/g)	809 ± 87	855 ± 49	NS	679 ±41	797 ± 60	NS

[a] All values are Mean ±SEM of six animals in each group.
[b] Student's *t* test. NS denotes "not significant", i.e. $P > 0.05$.

ities of this enzyme in the two subcellular fractions of the kidney extracts. Interestingly, no significant difference between M and XA line animals was observed in renal *N*-acetyl-β-D-hexosaminidase activity, in contrast to what was reported in the streptozotocin-diabetic rats which had significantly depressed level of this enzyme in the kidney (1). The cause for this discrepancy could arise from difference in species or between chemically-induced and hereditary diabetes.

Renal α-glucosidase activity was more prevalent in the 12,000*g* supernatant fraction, i.e. 73%, and no significant difference in its activity was observed between sexes or lines in either fraction. In preliminary experiments, β-glucosidase was found to reside mainly in the supernatant, i.e. 95%, and its activity was thus not measured in the precipitate fractions subsequently. Again, there was no significant difference in β-glucosidase activities between sexes or lines in the kidney.

Sex- and line-dependent differences in renal α-galactosidase and β-galactosidase activities, however, were highly significant in the supernatant fraction. Female animals showed higher activities in both enzymes than the male animals in either M or XA line. The diabetic XA animals also showed significantly less α-galactosidase and β-galactosidase in the kidney supernatant fractions than the corresponding M line animals with the following *P* values: <0.025 (α-galactosidase in female), <0.005 (α-galactosidase in male), <0.05 (β-galactosidase in male) and <0.005 (β-galactosidase in female). In the streptozotocin-diabetic rats, depression of renal β-galactosidase was also reported but the level of α-galactosidase has not been measured (1). The activities of these two enzymes in the 12,000*g* precipitate fractions were substantial but were not significantly different between sexes or lines.

Although the sex- and line-dependent variations in α-galactosidase and β-galactosidase appeared to be parallel, their activities were manifested by different proteins. When the 12,000*g* supernatant fraction of a M-line female animal was chromatogrammed on a Cellex-D column, β-galactosidase was eluted off in two early peaks,

whereas α-galactosidase was found to be more acidic at pH 6.0 and recovered in one major peak (Fig. 1). Therefore, the observed differences in α-galactosidase and β-galactosidase involved at least two to three protein entities. The observed sex- and line-dependent differences in these renal enzyme activities suggest that their levels may play significant roles in the development of diabetic microangiopathy in view of the fact that sex- and diabetes-related variations were reported in the width of muscle capillary basement membrane in humans (2). Studies to determine if the observed differences between the highly inbred lines were results of hyperglycemia or other genetic variants unrelated to hyperglycemia and if the differences between sexes or lines involved specific isozyme species are currently in progress.

Summary. Several glycohydrolase activities were measured in the 12,000*g* supernatant and precipitate fractions of kidney homogenates obtained from two highly inbred lines of male and female Chinese hamsters, one with hereditary diabetes. No difference was observed in *N*-acetyl-β-D-hexosaminidase, α-glucosidase and β-glucosidase whereas the activities of supernatant α-galactosidase and β-galactosidase were lower in the males and further depressed in the diabetic animals.

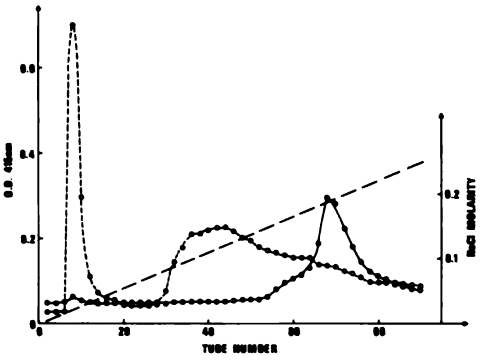

FIG. 1. Cellex D column chromatography of α-galactosidase and β-galactosidase in 12,000*g* supernatant fraction of Chinese hamster kidney extracts. Size: 0.9 × 30 cm. Fraction: 2.2 ml. ●——● = α-galactosidase. ○·····○ = β-galactosidase. ---- NaCl concentration in the eluting buffer.

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An Unusually High Divalent Cation Requirement for Attachment of West Nile Virus to Primary Chick Embryo Cells (40046)

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The *in vitro* assay of West Nile virus (WNV) has met with varying degrees of success in the hands of different investigators who have employed a wide variety of cell systems and experimental conditions. Henderson and Taylor (1) were unable to demonstrate either CPE or plaque formation in chick embryo (CE) cell cultures. However, Buckley (2) reported that WNV produced both CPE and plaques in CE cultures but the *in vitro* titers were considerably lower than those achieved in experimental animals. Similar results were obtained by Westaway (3) using PS cells (a stable line of porcine kidney cells). A wide variety of primary cell culture systems (1, 4–9) and continuous cell lines (2, 10, 11) have been employed to demonstrate the capacity of WNV to cause CPE and/or plaque formation. Most of these studies were more or less screening tests of a qualitative nature designed to demonstrate the ability of WNV to cause CPE or plaques rather than a systematic examination of conditions for a sensitive *in vitro* assay for WNV. The variations in CPE and plaque formation are understandable in view of the diverse experimental procedures employed. Little has been done to elucidate the conditions requisite for an accurate *in vitro* assay of WNV, although in a preliminary report Eylar and Wisseman (12) reported that the sensitivity of the WNV plaque assay was influenced by the pH of the adsorption diluent. This communication reports an unusually high divalent cation requirement for maximal attachment of WNV to primary CE cells.

Materials and methods. Virus. The Egypt 101 strain of WNV, which had been passed serially in mouse brains through an undetermined number of passages, was employed in this investigation. Stock virus, as 20% suckling mouse brain suspensions in 50% normal, heat-inactivated rabbit serum–saline with 250 units/ml penicillin and 125 μg/ml streptomycin sulfate, was stored in sealed glass ampoules at −70°.

Cell cultures. Primary CE cell cultures were prepared by the method of Dulbecco (13) with minor modifications. Trypsinized cells were suspended in 5% heat-inactivated calf serum–yeast extract medium (14) with penicillin and streptomycin added to a final concentration of 250 units/ml and 125 μg/ml respectively and, dispensed in 5 ml amounts into 60 × 30 mm plaque bottles (15).

In vitro assay. The growth medium was removed and the monolayers rinsed with Hanks' balanced salts solution (BSS). Unless otherwise specified, virus dilutions were prepared in borate–KCl buffer at pH 8.3–8.5 so that there were approximately 1000 PFU/ml. Four to six replicate bottles containing 0.1 ml inoculum were incubated at 26° with intermittent rocking for 90 min. In experiments where adsorption had to be interrupted prior to completion, adsorption was halted by addition of 3–4 ml Hanks' BSS, the virus–BSS mixture removed and the wash procedure repeated twice. Monolayers were overlayed with a medium consisting of 2% calf serum, 0.25% lactalbumin hydrolysate, BSS without phenol red and 0.8% noble agar. Sodium bicarbonate was added to a final pH of 7.3–7.4. Bottles were inverted and incubated at 37° for three days when a second overlay consisting of 2% noble agar in BSS with 1:20,000 neutral red was added. Plaques were counted after overnight incubation at 37°.

Results. Influence of divalent cation concentration on plaque count. During preliminary experiments in which diverse diluents were employed, it became apparent that components within the diluent markedly influenced the final plaque count. Since cations and especially divalent cations, are known to influence attachment of certain

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viruses to host cells (16–19), the possibility of a cation requirement in the WNV-CE cell culture system was investigated. Accordingly, plaque assays were performed with mixtures in which $MgSO_4$ at various concentrations was added to a constant amount of WNV in borate-KCl diluent at pH 8.3–8.5. The results of these experiments (Fig. 1) clearly show that the $MgSO_4$ concentration played an important role in the productive adsorption of WNV to CE cells. Little adsorption occurred when the $MgSO_4$ concentration was less than 1×10^{-4} *M*, but with increasing $MgSO_4$ concentration plaque counts increased to a maximum at approximately 0.1 *M* $MgSO_4$. Similar results were obtained when $MgCl_2$, $BaCl_2$ or $CaCl_2$ were employed. To determine if these results were influenced by anions and to determine the influence of monovalent cations upon WNV adsorption, similar concentrations of Na^+, K^+, Mg^{++}, Cl^- and $SO_4^=$ in various combinations were employed in the adsorption medium. Of these, only Mg^{2+} increased plaque counts. Several other cations were also tested (viz., Fe^{2+}, Al^{3+}, Sn^{2+}, Mn^{2+}) but, of all of the cations utilized, only Mg^{2+}, Ba^{2+} and Ca^{2+} significantly increased the plaque counts.

The influence of Mg^{2+} concentration on the rate of WNV adsorption to CE cultures. The time course of adsorption of WNV to CE cell cultures, at four different Mg^{2+} concentrations, was studied. Adsorption was allowed to continue for predetermined time intervals when the infected cultures were washed with BSS and overlaid with the agar medium.

Figure 2 shows the results of this study. Adsorption was most rapid when 0.1 *M* Mg^{2+} was incorporated in the virus inoculum, and adsorption was essentially complete within 60 min. If the Mg^{2+} concentration was suboptimal (less than 0.1 *M*), adsorption was much slower and maximal adsorption was not achieved in the 300-min time period studied.

Discussion. An unusually high divalent cation concentration, with activity restricted to certain members of the cation group, was found for optimal attachment of WNV to, and maximal assay sensitivity in, CE cells. A 200-fold increase in plaque count

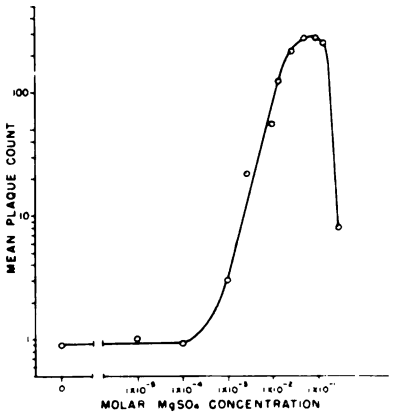

FIG. 1. The relationship between magnesium ion concentrations and relative WNV plaque count. A constant virus inoculum was mixed with different concentrations of magnesium sulfate in borate buffer and assayed in primary chick embryo cells.

from the same virus input occurred when the final Mg^{2+} concentration in the adsorption medium was raised from 1×10^{-4} *M* to 1×10^{-1} *M*. At approximately 1×10^{-3} *M* Mg^{2+} or Ca^{2+} (the approximate concentration of these ions in Hanks' BSS) there is minimal enhancement of WNV adsorption to CE cultures. This perhaps explains in part the varied results obtained when BSS or BSS containing diluents were employed in preliminary experiments. With BSS diluents, slight alterations in the cation concentration would result in exaggerated changes in the plaque count.

Influence of divalent cations on adsorption of viruses to host cells has been reported occasionally. For example, attachment of specific phages to *E. coli* (16), *Pasteurella* (17) or *Salmonella* (18) has a divalent cation requirement as does Japanese encephalitis virus to hamster kidney cells (19), and dengue virus to Rhesus monkey kidney cells (20). However, cation requirements are not necessarily uniform within a broad virus group. While added divalent cations did enhance the attachment of coxsackie A9 virus to human amnion cells (21), divalent cations were not essential for optimal attachment of poliovirus to HeLa cells (22). Attachment of Venezuelan equine encephalomyelitis virus to McCoy cells was optimal with 0.10–0.15 *M* NaCl while low levels of Ca^{2+} or Mg^{2+} inhibited

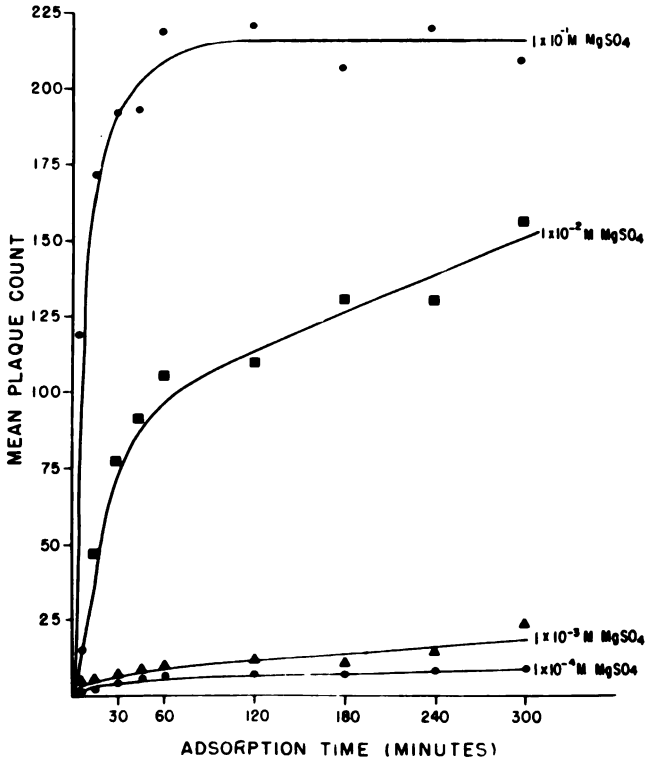

FIG. 2. Adsorption of WNV to chick embryo monolayer cultures from 0.1 ml inoculum at 26° at various $MgSO_4$ concentrations. Stock virus was added to borate-KCl buffer with 1×10^{-1} *M* $MgSO_4$ (●——●), 1×10^{-2} *M* $MgSO_4$ (■——■), 1×10^{-3} *M* $MgSO_4$ (▲——▲) and 1×10^{-4} *M* $MgSO_4$ (●——●). At the indicated time intervals replicate cultures were removed, adsorption halted by repeated washes with BSS and overlayed with agar.

virus attachment (23). Thus, cation enhancement of attachment of a virus to host cell most likely reflects a specific host cell-virus requirement rather than a general characteristic of the virus *per se*.

The enhanced WNV attachment to CE cells could not be attributed to (1) stabilization of WNV by Mg^{2+}, as has been demonstrated with the enteroviruses, because equivalent WNV stability was observed with or without 0.1 *M* Mg^{2+} (data not shown), (2) pH stability because the stability of WNV at pH 7.0 to pH 8.5 remained relatively constant (data not shown), (3) Mg^{2+} reversal of borate interactions with the carbohydrate component of virus or host cell glycoprotein (24) because a similar Mg^{2+} enhancement could be demonstrated when tris buffer was substituted for borate buffer, (4) increased virus yield as a result of promoting migration of viral nucleocapsids to the cell membranes (25) because increased Mg^{2+} levels were required for the virus attachment process only. In addition it was found that increased levels of Mg^{2+} enhanced WNV attachment to BHK-21/15 cells but not to the extent observed in the CE cell-WNV system.

Thus, the divalent cation requirement for optimal attachment of WNV to CE cells appears to be unrelated to pH stabilization, Mg^{2+} stabilization, buffer effects, virus replication and assembly or to the source of the host cell. It is more likely that Mg^{2+}, and to a lesser degree Ca^{2+} and Ba^{2+} serve as a mechanism by which the negatively charged virus and host cell can come into intimate contact to initiate the adsorption and penetration process.

Summary. An unusually high divalent cation requirement for maximal plaque count and maximal adsorption rate was found in the WNV-CE cell system. An increase in Mg^{2+} concentration from 1×10^{-3} to $1 \times$

0^{-1} *M* resulted in a 200-fold increase in verage plaque count. When 1×10^{-1} *M* lg^{2+} was incorporated in the virus inocuım, WNV attachment was essentially comlete in 50–60 min. If 1×10^{-2} *M* or less lg^{2+} was included in the adsorption dilunt, maximal attachment was not complete ithin 300 min and only a fraction of the riginal virus content could be detected by laque assay.

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Epstein-Barr Virus Infection of Cryopreserved Umbilical Cord Blood Lymphocytes (40047)

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Infectious Epstein-Barr virus (EBV) will infect and transform lymphocytes from several primate species (1-4). The most sensitive primary culture system for the detection of infectious EBV is human neonatal lymphocytes, usually obtained from umbilical cord blood specimens (5, 6). Each milliliter of umbilical cord blood will yield up to 3×10^6 lymphocytes; from a single cord blood specimen, 10^8 lymphocytes or more can be obtained. Cells to be infected may be kept at 37° in appropriate medium for several days before being used, but eventually, through attrition, become unsuitable for use. Generally, an investigator obtains a fresh supply of cells each time EB virus infection studies with primary lymphocytes are undertaken. The availability of a cell storage method which preserves cells, in a state comparable to fresh cultures, would remove one of the major limitations concerning studies of the biology of primary lymphocytes infected with EBV.

Cryopreservation of tissue culture cells has been described and practiced for years with monolayer cell lines (7). Freezing of primary leukocytes has likewise been described (8-10) but we are unaware of studies which compare EBV transformation sensitivities of cryopreserved lymphocytes with fresh lymphocytes. We here describe preservation of lymphocytes in the vapor phase of liquid nitrogen and present data indicating that, compared to autologous fresh cells, cryopreserved cells remain sensitive to infection and transformation with EB virus.

Materials and methods. Cell collection and preparation. Neonatal lymphocytes were obtained by venipuncture of recently delivered placentas (up to 30 min post delivery). Whole blood was mixed with preservative-free sodium heparin (10 units/ml blood) to prevent coagulation. Lymphocytes were separated in a Ficoll-hypaque gradient (11), washed free of plasma in three 1:5 volumes of Puck's saline A and counted in 1% acetic acid. Cells for fresh culture were adjusted to 5×10^5 cells/ml in maintenance medium; cells for cryopreservation were adjusted to 1×10^7 cells/ml in freeze medium.

Maintanence medium consisted of RPMI 1640 supplemented with 20% heat inactivated (56°, 30 min) fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). Freeze medium consisted of RPMI 1640, 20% heat inactivated fetal bovine serum and 10% analytical reagent grade dimethyl sulfoxide (DMSO) (Mallinckrodt Chemical Works).

Feeder layers used in the transformation assay consisted of cell strains of human placental fibroblasts, established as described by Miller (12). Assays were carried out in maintenance medium.

EBV preparation. Virus stock was prepared from supernatant fluid of an EBV-transformed marmoset cell line (B95-8) (2). Fluid was centrifuged at 400*g* to sediment cells, frozen and thawed three times and passed through a 0.45 nm filter (Millipore Corp.). The virus stock was determined to be free of bacteria and mycoplasma by culture, and was stored at −70°.

Cryopreservation. Lymphocytes suspended in 1 ml of freeze medium and transferred to plastic vials (Cooke Pro-vial) were placed in a polystyrene plug (Linde biological freezer, type BF-5, Union Carbide Corp.) fitted to the neck tube of a liquid nitrogen refrigerator (type LNR25B, Union Carbide Corp.) (13). By placing the "O" ring on the plug at level "F", the temperature decrease was constant at 1.6° per min until the sample changed from a liquid to a

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solid (18 min) after which the temperature decrease followed a sigmoid curve reaching −50° by 60 min. The vials were then stored at −170° in the vapor of a liquid nitrogen storage tank (type LR-36, Union Carbide Corp.).

In experiments depicted in Tables I and II, lymphocytes were stored frozen overnight. Cell stocks for other experiments (Table III) were stored frozen up to 12 months.

Virus inoculation and transformation assay. Frozen lymphocytes were rapidly thawed (37° water bath) and resuspended in medium (5×10^5 cells/0.9 ml). Fresh lymphocytes, stored in medium overnight at 37° in 5% CO_2, were resuspended at a concentration of 5×10^5 cells/0.9 ml. Both culture sets were exposed to serial tenfold dilutions of EBV (0.1 ml), or to culture medium (0.1 ml), four replicates per set, and cultivated in combination with human placental fibroblast feeder layers in the bottom of plastic capped, 13 × 100 mm glass test tubes. Medium was changed (1/2 vol) twice weekly and cultures were examined with the aid of an inverted microscope for signs of cell transformation. Transformation was recognized by the appearance of clusters of rapidly growing, enlarged lympho-

TABLE III. Repeated Titrations of a Single Stock Preparation of EBV[a] on Cryopreserved Human Umbilical Cord Blood Lymphocytes.

Cord blood preparation	Feeder layers[b]	Duration of cryopreservation (days)	Infectivity titer[c]
1	Yes	23	4.0
2	Yes	360	3.5
3	Yes	1	3.0
4	Yes	1	3.0
5	Yes	1	3.5
5	No	1	2.8
6	No	17	1.8
7	No	1	2.6

[a] Stock prepared from supernatant fluid of EBV infected cotton-top marmoset lymphocytes (B95-8), stored at −70° until used.
[b] Human placental fibroblasts (see Ref. 12).
[c] Expressed as 50% transforming doses ($\log_{10}TD_{50}$) per 0.1 ml, calculated by Reed-Muench formula.

TABLE I. Comparison of EB Virus Transforming Titers Detected by Fresh or Autologous Cryopreserved Lymphocytes.

	Sample A[b]		Sample B		Sample C		Cumulative	
Virus dilutions[a]	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
10^{-2}	3/4[c]	4/4	4/4	4/4	4/4	4/4	11/12	12/12
10^{-3}	1/4	3/4	1/3	2/4	3/4	1/4	5/11	6/12
10^{-4}	0/4	0/4	1/4	0/4	1/4	2/4	2/12	2/12
10^{-5}	0/4	0/4	0/4	0/4	0/4	0/4	0/12	0/12
nfectivity titer[d]	2.5	3.3	3.0	3.0	3.5	3.0	3.0	3.2
No virus	0/4	0/4	0/4	0/4	0/4	0/4	0/12	0/12

[a] Aliquots from a single virus stock (B95-8, lot #5) used throughout these experiments.
[b] Human cord blood lymphocytes from three separate placentas designated A, B, and C. In each sample cells were either frozen overnight (frozen) or incubated overnight (fresh) before infection with EBV.
[c] Number of cultures showing transformation/number of cultures in test.
[d] Expressed as 50% transforming doses ($\log_{10}TD_{50}$) per 0.1 ml, calculated by Reed-Muench formula.

TABLE II. Comparison of EB Virus Transforming Titers Detected by Fresh or Dimethylsulfoxide (DMSO) Treated Fresh or Cryopreserved Lymphocytes.

		Virus Dilution					
Condition of cells[a]	Percent DMSO	No Virus	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Infectivity titer[c]
Fresh	none	0/4[b]	4/4	3/4	1/4	0/4	3.5
Fresh	1	0/4	4/4	4/4	1/4	0/4	3.7
Fresh	10	0/4	4/4	4/4	0/4	0/4	3.5
Frozen	10	0/4	4/4	1/4	2/4	0/4	3.0

[a] Human cord blood lymphocytes from sample C, Table I. Cells were either cryopreserved overnight (frozen) or incubated overnight (fresh) before infection with EBV.
[b] Number of cultures showing transformation/number of cultures in test.
[c] Expressed as 50% transforming doses ($\log_{10}TD_{50}$) per 0.1 ml, calculated by Reed-Muench formula.

cytes. Endpoint for transformation was determined 60 days after cultures were initiated. Infectivity titers were calculated by the Reed-Muench formula (14) as a function of the number of culture tubes showing transformation compared to the number inoculated with virus.

Results. Transformation of lymphocytes before and after cryopreservation. In two experiments, using cord blood lymphocytes obtained from three separate placentas, comparisons were made between autologous lymphocyte sets which had been either cryopreserved overnight or were incubated fresh overnight (Table I). Serial dilutions of virus from a single virus stock yielded transformation endpoints ranging between $10^{2.5}$ TD_{50}/0.1 ml (TD = transforming dose) and $10^{3.5}$ TD_{50}/0.1 ml in the three fresh lymphocyte sets and between $10^{3.0}$ TD_{50}/0.1 ml and $10^{3.3}$ TD_{50}/0.1 ml in the three cryopreserved lymphocyte sets. Cryopreserved lymphocytes are as sensitive to transformation by EBV as unfrozen, fresh cultured lymphocytes. Neither fresh nor cryopreserved lymphocytes transformed unless EBV was added. Cell viability of recovered cryopreserved lymphocytes ranged between 85–88% by trypan blue dye exclusion.

Transformation of fresh lymphocytes exposed to DMSO before infection with EB virus. To establish whether preincubation of lymphocytes with DMSO could alter the sensitivity of cell cultures to transformation with EB virus, fresh cells from cord blood sample "C" were incubated with 1% or 10% DMSO for one hour prior to exposure to EB virus. Table II shows that preexposure of lymphocytes to two concentrations of DMSO neither enhanced nor suppressed the sensitivity of lymphocytes to EBV transformation.

Reproducibility of the transformation assay using cryopreserved lymphocytes. Over a three year period, we have stored, by cryopreservation, thirty samples of umbilical cord blood lymphocytes prepared as described. Titrations of a single lot of EBV have been performed on seven different cryopreserved umbilical cord blood lymphocyte samples. Table III shows that the infectivity titer on cryopreserved cells varies according to the presence of feeder layers; cultures with feeder layers detected tenfold more virus than cultures without. However, infectivity titers remain relatively reproducible from sample to sample of cord blood lymphocytes. The longest cryopreservation period for a specific cord blood preparation was 12 months; when thawed that sample's virus sensitivity detected an infectivity titer of $10^{3.5}TD_{50}$/0.1 ml (preparation 2, Table III).

Transformation of adult lymphocytes. The cryopreservation technique has been applied to lymphocytes from sources other than cord blood. Although, we have not compared infectability of cryopreserved cells with fresh cells, cryopreserved lymphocytes from adult humans and cotton-top marmosets can be readily transformed and will support EB viral replication. Cryopreserved lymphocytes from EBV-antibody negative adult sources, cultured without addition of EB virus, have not transformed.

Discussion. Considerable experimental evidence indicates that the efficiency of recovery of cells after cryopreservation depends not only on the rate of temperature change and the cryoprotective additives used, but also on the type of cell being frozen (for reviews see 15, 16). Cells recovered by Ficoll-hypaque gradient separation of heparinized peripheral blood are a heterogenous collection of mononuclear cells containing not only morphologically different cells (lymphocytes and monocytes) but also cells which, although morphologically quite similar, carry a wide variety of membrane components as well as different biologic functions. EB virus infects lymphocytes with B-cell surface characteristics (17). While EB virus receptors have been demonstrated to be common to all B-lymphocytes (18), studies of the efficiency of transformation in B-lymphocyte enriched cultures, indicate that only 10% of the virus exposed cells establish permanent lines (6). Thus, there appears to be a very small subpopulation of cells in each sample of peripheral blood lymphocytes destined for transformation induced by EB virus.

The presently reported studies were undertaken to establish that even though peripheral blood lymphocytes can be preserved through freezing with relatively high

ıcy, the small, and as yet unidenti-
opulation of cells which are the target
V infection and transformation, can
ɜ adequately preserved by this freez-
ocedure. Our studies indicate that,
red to autologous fresh cells, cry-
rved lymphocytes remain sensitive to
on and transformation by EB virus.
ıethyl sulfoxide, used here as a cry-
ctive additive, is also known to en-
the transport of infectious nucleic
cross cell membrane (19). Since we
ı enhancement of virus induced trans-
ːion when fresh lymphocytes were
ated with DMSO, we conclude that
milarity of infectivity titers seen in
eserved cells and fresh cells is due to
yoprotective effect of the chemical,
enhanced transport of viral informa-
ːross the target cell membrane.

er and Lipman (20) showed good
lucability of the transformation assay
ctious EBV using fresh primary cord
lymphocytes. We here present com-
e data on cord blood lymphocytes
ːryopreservation. Recently, Hender-
al. (6) have shown that EB virus
ɔn, using fresh lymphocytes cocultı-
with feeder layer tissues, leads to
ːed sensitivity of the transformation
Our data using cryopreserved lym-
ːes (Table III) supports that concluı-
ymphocytes cocultivated with feeder
resulted in approximately tenfold
ˈirus transformation activity than lym-
ːes cultured without feeder layers.

technology required to freeze and
ɔrimary umbilical cord blood lympho-
s simple to apply, has been extended
ude peripheral blood lymphocytes of
human and nonhuman primates and
ɪ to major reduction in time spent in
ːy rooms waiting for umbilical cord
specimens. In addition, large samples
phocytes from the same infant can be
ed, preserved and referred to as often
ded, making possible repeated inves-
ns on the same sample of lymphocytes
long series of experiments. Finally,
a cell system sensitive to EBV infec-
eserved for use whenever needed has
ːed the flexibility of the clinical diag-
ˈirology laboratory which is now able
to process specimens for isolation of EB virus as they sporadically arrive.

Summary. We have described the application of cryopreservation technology to freeze storage of human primary umbilical cord blood lymphocytes and have shown that the cryopreserved cells remain as sensitive to EB virus infection and transformation as the fresh autologous lymphocytes. The methodology has been satisfactorily applied to primary peripheral blood lymphocytes from adult human sources, and has been a convenient time and resource saving advance.

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Immunogenicity and Paralytogenicity of Antigens Solubilized from Line I_b Malignant Lymphocytes[1] (40048)

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When young C58 mice (<6 month-old) are immunized (1) to syngeneic line I_b malignant lymphoid cells (I_b cells) they are immune to a challenge to 10^7 viable cells. Immunity persists for at least a year (1) and is primarily T cell mediated (2). However, when young immunosuppressed C58 mice (3), or those immunologically compromised by age (4), are similarly immunized they develop a paralytic central nervous system disease (immune polioencephalomyelitis or IPE) that is characterized by an inflammatory destruction of neurons in the cord and brain stem (5). Current evidence indicates that as C58 mice age they spontaneously lose a T cell subpopulation resistant to 350R (6) that protects them from induction of the disease. We are interested in the central question of whether the I_b cell antigen that immunizes C58 mice to transplantable leukemia is also the one that elicits IPE. As a first step we compared the relative effectiveness of low energy sonication and 3 *M* KCl extraction for preparing solubilized antigens. We report here that antigens solubilized from I_b cells by either method elicited IPE in indicator C58 mice and immunized immunocompetent animals to the disease. Crude solubilized preparations also immunized mice to transplanted line I_b leukemia.

Methods. C58 mice. The linage of our strain of C58 mice (C58/wm) and the methods of mouse husbandry were described (1, 7). X-Irradiated 5 month-old, or non-x-irradiated 9-12 month-old mice, were used for assays of IPE (3). *I_b cells.* The methods used to prepare suspensions of viable I_b cells were described (1, 2). Spleens removed from mice moribund from transplanted leukemia were dispersed gently in cold (4°) HBSS (Hanks balanced salt solution) and teased apart with scalpels to yield single cell suspensions. Cell preparations were allowed to stand 3-5 min to let gross particles settle. The supernatant cells were transferred to a conical centrifuge tube and sedimented at 250*g* for 3 min. The sedimented cells were washed three times and resuspended in 10 ml of HBSS. Total and viable cell counts (trypan blue exclusion) were done as described previously (1, 2). Unless specified otherwise suspensions contained *ca.* 10^8 viable cells/ml.

X-Irradiation. X-Irradiated I_b cells were used to elicit IPE (3). I_b cells were irradiated in a 60 × 15 mm plastic dish containing 5-10 ml of cell suspension. Cells were irradiated by a Westinghouse Coronado X-ray Therapy Machine operated at 200 KV and 15 ma through a 1.0 mm Al filter to produce a dose of 527.5 R/min at a target distance of 24 cm. A dose of 10,000 R was used. Five month-old C58 mice used for IPE assays received 525 R with the machine calibrated to deliver 67 R/min at a target distance of 70 cm when operated at 250 KV and 15 ma using 0.5 mm Cu and 1.0 mm Al filters. One day after x-irradiation they were challenged by the ip injection of 10^2 or 10^3 x-irradiated I_b cells.

Assays for IPE. The main clinical and histopathologic features of the disease and the statistical limits of assays, were described (3). The earliest onset occurs 9-10 days after the ip injection of 10^3 x-irradiated I_b cells with a peak at 12-15 days. Since IPE may develop as late as 20-30 days all mice were scored over a 30-day observation period.

Sonication. Care was taken to employ the optimum conditions described by Reisfeld and Kahan (8). Viable cells were sonicated for 5 min (10 KHz @ a.25 ma, 4°) using a Raytheon low intensity DF101 oscillator.

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rganelles and debris were sedimented
quential centrifugation: 600 × g for
in followed by 30,000*g* for 60 min.
esultant supernatant fluid was labeled
This preparation then was centrifuged
),000*g* for 2 hr at 4° and designated
. The 100-s preparation was dialyzed
it 100 vol of PBS at 4° over a 24-hr
l. PBS was changed four times at 6-hr
als. The product was designated 100-
.ll preparations were passed through a
.m filter (Millipore Corporation, Bed-
MA) and stored at −60°.

1 KCl extraction. The methods were
ed from those described by Metzler *et*
). Seven milliliter of a solution contain-
.27 *M* potassium chloride and 0.005
tassium phosphate buffer, pH 7.4 was
l to 3 ml (4°) of cell suspension. After
ation for 18 hr the solution was clari-
y centrifugation at 30,000*g* for 1 hr.
ellet was discarded and the superna-
luid was dialyzed against two changes
) vol of PBS at 4°. The slight precipi-
hat formed was removed by centrifu-
l at 30,000*g* for 60 min. The product
lesignated 30-k. Further centrifuga-
filtration, and dialysis were the same
scribed above thus yielding prepara-
designated 100-k and 100-k,d, respec-
.

hadex filtration. Solubilized antigen
rations were fractionated by column
natography employing either Sephadex
) or Sephadex G-200 (Pharmacia Fine
icals; Uppsala, Sweden). Samples
fractionated on 0.9 × 30 cm columns.
alf milliliter fractions were collected
30 min. Columns were eluted with
M sodium phosphate buffer with 0.10
dium chloride, pH 7.4 at 4°. Protein
ntrations were determined by the
od of Lowry *et al.* (10). All columns
calibrated with blue dextran (mol wt
^{6}), bovine serum albumin (mol wt =
0), and egg white lysozyme (mol wt
,800).

ctron microscopy. At various stages of
n preparation samples were examined
negative staining technique. Droplets
lubilized preparations were deposited
rmvar carbon coated, two-hundred
, grids. After blotting specimens dry
were negatively stained with 2% phos-
photungstic acid (aqueous) for 30 sec. Samples were examined in a JEOL-100B electron microscope (Medford, MA) operating at 60 KV.

Results. Solubilization of antigens. Antigens were solubilized from I_b cells by either low frequency sonication (5 min, 10 KHz, 1.25 ma, 4°) or 3 *M* KCl extraction (18 hr, 4°) as described. Decreases in protein concentration during fractionation were compared using the starting preparations 30-s and 30-k as the reference. With the sonicates there was approximately a 12% decrease in protein between the 30-s and 100-s preparation with an additional 17% decrease after dialysis. Overall, there was approximately a 27% decrease between the 30-s and 100-s,d products. For the KCl extracts the decrease in protein concentration between the 30-k and 100-k preparation was approximately 7% followed by an additional 28% decrease after dialysis. Overall, there was a 29% decrease in protein content between the 30-k and 100-k,d products. Electron microscopic examination of the various preparations showed that the 30-s preparation contained numerous cell membrane fragments; C-type particles were rare. Corresponding 30-k preparations did not contain detectable C-type virus particles and membrane fragments were rare. The 100-s preparations did not contain detectable C-type virus particles; membrane fragments were rare. The 100-k preparations did not contain detectable membrane fragments or C-type virus particles.

Sephadex G-150 gel filtration. These experiments were done to determine whether solubilized preparations were stable, recoverable from gels, and to indicate the major molecular species that were immunogenic or paralytogenic. The 30-s preparation containing three major molecular species (Fig. 1A). Peak 1 (tubes 15–21) consisted mainly of the void volume, mol wt ≥ 150,000. Peaks 2 (tubes 22–33) and 3 (tubes 34–39) had mol wt of approximately 69,000 and 14,000, respectively. The 30-k preparation contained only 2 major molecular species corresponding to mol wt of approximately 60,000 (tubes 25–33) and ≤14,000 (tubes 34–40), respectively. The slight shoulder (tube 21) in the first peak suggested the occurrence of a third unre-

solved molecular species. When the 30-s preparation was ultracentrifuged at 100,000*g* for 2 hr (Fig. 1B), the profile for the 100-s preparation was essentially the same as in Fig. 1A except for lesser amounts. However, four peaks were found in the 100-k preparation. Figure 1C shows that dialysis removed the lower molecular weight species from both the 100-s and 100-k preprations.

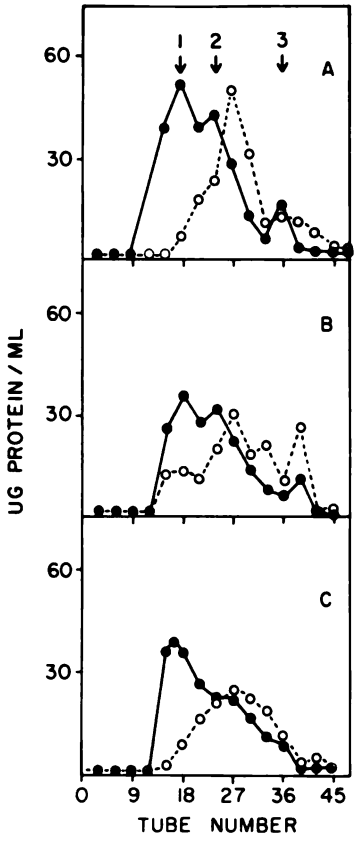

FIG. 1. Sephadex-150 gel filtration of solubilized preparations. Columns (0.9 × 30 cm) were equilibrated with 0.05 *M* sodium phosphate buffer containing 0.10 *M* sodium chloride, pH 7.4. Columns were calibrated (arrows, left to right) with the dextran (mol wt, 2 × 10^6), bovine serum albumin (mol wt, 69,000) and egg white lysozyme (mol wt, 14,000). Each column received 0.3 ml of preparation (0.725 mg protein/ml); 0.5 ml fractions were collected every 30 min (4°) by elution with the described buffer. The Lowry method (10) was used for protein determinations. (A) shows 30-s (●——●) and 30-k (O- - -O); (B) 100-s (●——●) and 100-k (O- - -O); and (C) 100-s,d (●——●) and 100-k,d (O- - -O).

Induction of IPE by solubilized preparations. Table I shows that the 30-s and 30-k preparations were fully paralytogenic at ≥1 μg. The 100-s, 100-k, 100-s,d, and 100-k,d paralyzed about 50% of mice at the 100 μg level but not at the 1 μg concentration. Control preprations from normal C58 mouse spleens, or from spleens of mice with spontaneous leukemia, were not paralytogenic. Table II shows that preparations eluted from Sephadex G-150 columns elicited IPE.

Immunization of mice to IPE. Three-month-old normal C58 mice received from 1 to 4 weekly injections ip of solubilized preprations. Four weeks later mice were tested for their capacity to resist the induction of IPE when challenged by the injection of x-irradiated I_b cells. Control mice received either HBSS or normal syngeneic spleen cells in place of the immunizing preparation. Table III shows that all test preparations were immunogenic.

TABLE I. INDUCTION OF IPE BY SOLUBILIZED LINE I_b CELL PREPARATIONS.

Cell preparation	Protein (μg) injected ip	Fraction of mice that[a] developed IPE
30-s	100	5/5
	10	8/8
	1	5/7
30-k	100	7/9
	10	7/9
	1	8/8
100-s	250	15/17
	100	3/8
	1	1/9
100-k	250	4/4
	100	5/7
	1	0/7
100-s,d	250	9/9
	100	6/10
	1	0/10
100-k,d	250	4/4
	100	6/9
	1	0/10
NMS-30-s[b]	250	0/19
SL-30-s[c]	500	0/5

[a] Number of mice with IPE/number injected. Five-month-old indicator mice received 525R 1 day before ip injection of 1 ml of each preparation. Mice were scored for paralysis over a 30-day period.

[b] Normal mouse spleen cells were used in place of I_b cells.

[c] Spleen cells were obtained from C58 mice (6-12 months old) with spontaneous leukemia.

E II. INDUCTION OF IPE BY 30-s AND 30-k ICTIONS ELUTED FROM SEPHADEX G-150 COLUMNS.

ıate[a]	μg or ng of protein injected ip	Fraction paralyzed[b] 30-s	30-k
	25 μg	16/16	Not done
= 150,000)	2.5 μg	14/15	
	250 ng	10/15	
	25 ng	4/16	
	2.5 ng	0/14	
	25 μg	10/20	12/21
= 69,000)	2.5 μg	0/13	3/22
	250 ng	1/14	0/16
	25 ng	0/12	0/17
	2.5 ng	0/17	0/17
	25 μg	9/18	Not done
= 14,000)	2.5 μg	0/16	
	250 ng	0/16	
	25 ng	0/14	
	2.5 ng	0/13	
	250 μg	23/25	
ɔnated)		0/22	

ıtes from five tubes of the central portion of k were combined.
footnote a, Table I.

E III. IMMUNIZATION OF C58 MICE TO IPE SOLUBILIZED I_b CELL PREPARATIONS.

lized cell aration	Number of weekly[a] i.p. injections	Fraction of mice that developed IPE[b]
l-s	4	0/10
	3	0/10
	2	3/8
	1	2/10
l-k	4	1/9
	3	0/7
	2	0/5
	1	1/9
)0-s[c]	1	0/10
)0-k	1	0/10
SC[d]	1	12/20
BSS	1	13/24

ı 1 ml injection contained 1 mg of protein.
footnote a, Table I. Data were compiled from cate experiments.
: in the 100-s or 100-k groups received a ection 2 weeks before challenge.
adiated normal spleen cells were used for ation.

ınization to transplantable leukemia. n be immunized to purified I_b cell ınes (11) providing that optimum gimens are used. Immunization was fective when 10^3 viable I_b cells were l with purified membrane fractions. ar approach was used to determine the immunogenicity of the solubilized line I_b cell antigens. Table IV shows that 1–10 mg of 30-s or 1–5 mg of 30-k were immunogenic when injected with either 10^2 or 10^3 viable I_b cells. When mice received 2–3 ip injections of 30-s alone, spaced 4 days apart, and were challenged with 10^3 viable I_b cells 15 days later, 8/20 survived challenge. However, neither the 100-s or 100-k preprations were immunogenic as tested (Table IV).

Discussion. These experiments compared the effectiveness of solubilizing antigens from line I_b cells by low energy sonication and 3 *M* KCl extraction. The immediate objective was to determine which technique was superior and whether the products obtained elicited IPE or were immunogenic. The more long term objective is to determine whether the same molecular species of antigen that elicit IPE also are immunogenic to IPE and transplanted line I_b leukemia. In assessing results it is relevant to consider an appropriate definition of the term "soluble." Reisfeld and Kahan (8) suggested that solubility be defined to mean that preprations are free of membrane fragments as determined by electron microscopy, that they penetrate into chromatographic gels, and that they do not lose antigenic activity after "extensive ultracentrifugation at 130,000*g*." We found membrane fragments, and the C-type virus par-

TABLE IV. IMMUNIZATION OF C58 MICE TO TRANSPLANTABLE LEUKEMIA.

Solubilized preparation	Admixture[a] Mg solubilized antigen	Viable I_b cells	Survivors[b]
30-s	10	10^3	13/16
30-s	5	10^3	9/10
30-s	5	10^2	9/10
30-s	1	10^2	8/10
30-k	5	10^3	3/3
30-k	1	10^2	3/15
100-s	5	10^3	0/16
100-k	5	10^3	0/7

[a] Mice that received a single ip injection of each admixture were challenged with either 10^2 or 10^3 LD100 of viable I_b cells.

[b] Number of mice that survived immunization and challenge/number tested. When control mice received 10^7 viable normal C58 spleen cells in place of the soluble antigen none survived the immunization.

ticles associated with line I_b cells, to be useful markers to monitor purification by electron microscopy. Interestingly, the 3 *M* KCl preparations were essentially free of membrane fragments after centrifugation at 30,000*g* for 1 hr and C-type particles were not detected. Sonicates were not free of membrane fragments even after centrifugation at 100,000*g* for 2 hr. Metzgar *et al.* (12) have shown that centrifugation at 100,000*g* for 4–6 hr sediments membrane vesicles in KCl extracts of human leukemic cells. Such results suggest that it is difficult to free solubilized preparations from membrane fragments. The current results suggest that 3 *M* KCl extraction is a superior approach in this regard.

Within the context of the current studies the Sephadex gel filtration experiments were not carried out in sufficient detail to permit firm conclusions to be drawn concerning the precise molecular species possessing biological activity. It is clear, however, that the procedures used for purification did not destroy the biological activity of the various products. The Sephadex G-150 gel filtration data indicate that the high molecular weight "excluded volume" seen with sonicated preparations was greatly diminished in the 3 *M* KCl extracts. Several lines of evidence suggest that paralytogenic activity was not exclusively associated with the void volume. Table II presents data indicating that IPE was elicited by molecular species found in peak 2 (mol wt $\approx$ 69,000) and 3 (mol wt $\approx$14,000). These results confirm our earlier work (13) which showed that serum, obtained from mice 1 day after the ip injection of 10^7 inactivated I_b cells, contained molecular species of a size range of 64,000–165,000 that elicited IPE. Considered together the results indicate that molecular species with a size range less than 150,000 daltons may elicit the disease. From the current studies it appears that by the use of 3 *M* KCl extracts and preparative gel electrophoresis it should be possible to identify definitively the molecular species responsible for eliciting IPE. In this regard one key advantage of the IPE model is that the exquisite sensitivity of the assay for the disease makes it easy to trace and quantify biological activity during various steps of purification.

Since sensitive methods were worked (1-3) out for immunizing C58 mice to line I_b leukemia and IPE it was possible to test whether immunogenicity was lost during solubilization and purification of antigens. Table III shows that a single ip injection of 1 mg of the 30-s, 30-k, 100-s or 100-k preprations immunized mice to IPE. Table IV shows that the 30-s and 30-k preparations also immunized mice to transplantable leukemia but that the 100-s and 100-k preparations were not immunogenic under the simple test conditions that were used. Considered together, however, the results presented in Tables III and IV provide a basis for determining whether the same molecular species can be both immunogenic and paralytogenic in the line I_b-C58 model. The crucial difference in a protective response, compared with a paralytogenic one in the IPE model, appears to be governed (6) by a loss in a 350R resistant T cell subpopulation (presumably a suppressor T cell) which is lost spontaneously as C58 mice age.

Summary. The relative effectiveness of sonication and 3 *M* KCl extraction for solubilizing antigens from line I_b malignant lymphocytes were compared. Electron microscopic studies provided evidence that 3 *M* KCl extracts contained fewer membrane fragments and more closely approximated a rigorous definition of "soluble." Sephadex G-150 gel filtration data indicated that the solubilized preprations contained three main peaks, viz, the void volume, a molecular species $\leq$69,000 and a molecular species $\leq$14,000. Fractions eluted from Sephadex columns elicited immune polioencephalomyelitis in indicator mice. Solubilized preparations immunized mice to the disease. Preparations containing membrane fragments immunized C58 mice to transplantable line I_b leukemia.

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by JOHN PAPACONSTANTINOU

irty-Fourth Symposium of The Society for Developmental Biology, Orono, Maine, June 1-4, 1975

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Glycoproteins and proteoglycans
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References
Index

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calorie	cal	millimeter	mm
centimeter	cm	milliosmole	mOsm
counts per minute	cpm	minute	min
cubic centimeter	cm^3	molal (concentration)	*m*
Curie	Ci	molar (concentration)	*M*
degree Celsius (Centigrade)	°C	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	diam	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	*N*
inch	in.	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
liter	spell out	second	sec
meter	m	specific activity	sp act
microliter	µl	square centimeter	cm^2
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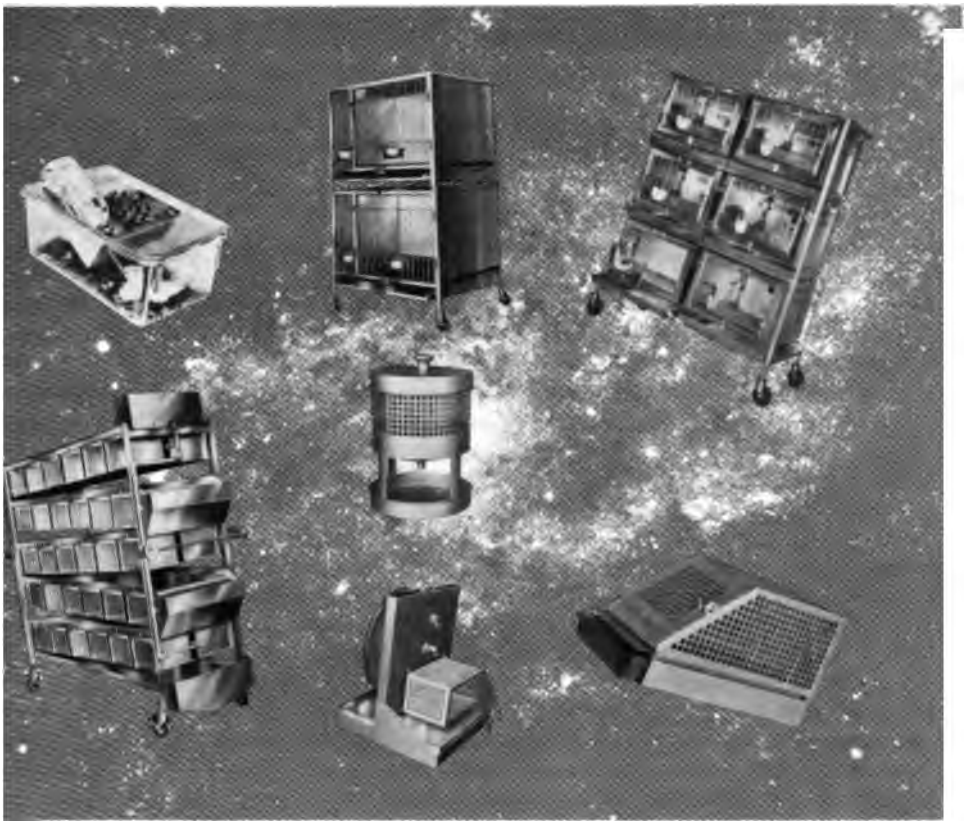

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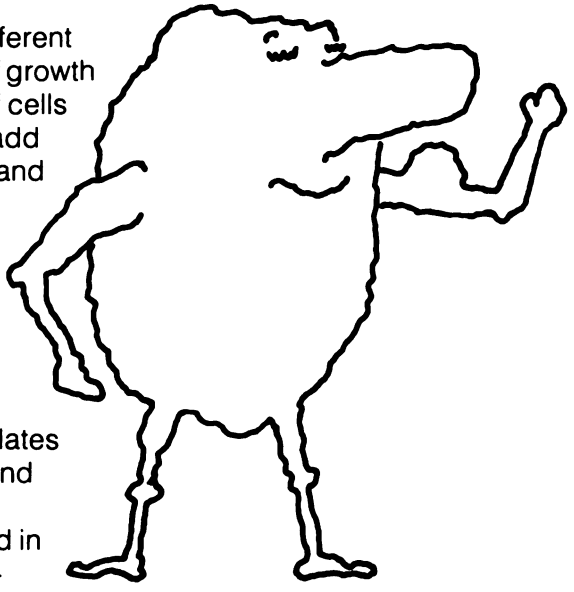

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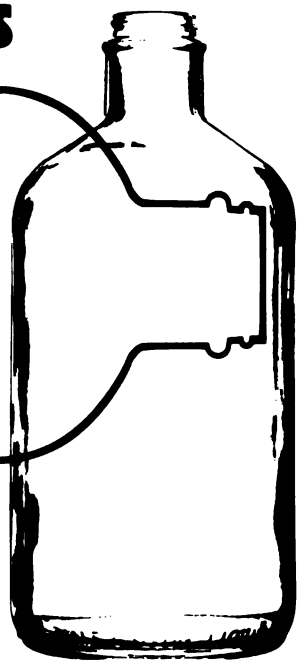

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Charles River ..... Cover 4

Collaborative Research Inc. v

Wahmann Manufacturing i

Winscot, Inc. vi

TABLE OF CONTENTS

BIOCHEMISTRY

Biochemical Properties of Bovine Granulocytes	R. Gennaro, C. Schneider, G. de Nicola, F. Cian, D. Romeo	342
Biosynthesis of Insoluble Elastin in Hamster Lungs during Elastase-Emphysema	S. Y. Yu, N. R. Keller, A. Yoshida	369
Collagen Composition of the Skin from Obese and Lean Zucker Rats	J. W. Coffey, V. J. Mattaliano, C. Fiedler-Nagy, A. C. Sullivan	435
Glycosaminoglycans of Cardiac Tumors	E. R. Dalferes, B. Radhakrishnamurthy, G. S. Berenson	461
Loss of Hepatic Monoamine Oxidase Activity Resulting from Replacement of its Coenzyme Flavin	Y. S. Kim, J. P. Lambooy	466

ENDOCRINOLOGY

Increased ATPase and Decreased Alkaline Phosphatase Activities by Parathyroid Hormone in Cultured Chick Embryo Tibiae	M. L. Thomas, W. K. Ramp	358
Hypocalcemia and Increased Serum Calcitonin in Baby Rats Given Glucose Orally	C. W. Cooper, J. F. Obie	374
Glucose Metabolism in Obese Men: Abnormal Intravenous and Normal Oral Glucose Tolerance	C. Abraira, A. M. Lawrence	407
The Influence of Adrenalectomy and of Corticosterone Administration on the Ether-Induced Increase in Plasma Prolactin in Ovariectomized Estrogen-Treated Rats	M. G. Subramanian, R. R. Gala	415
Inhibition of Oxytocin Release by Morphine and its Analogs	J. Haldar, W. H. Sawyer	476
Serum LH Levels Following Multiple LHRH Injections in Aging Rats	A. E. Miller, G. D. Riegle	494

GROWTH AND DEVELOPMENT

Oxygen Consumption and Body Fat Content of Young Lean and Obese (OB/OB) Mice	G. A. Boissonneault, M. J. Hornshuh, J. W. Simons, D. R. Romsos, G. A. Leveille	402

HEMATOLOGY

The Kinetics of Serum Ferritin	A. S. Pollock, D. A. Lipschitz, D. Cook	481
Intracellular Calcium: Lack of Effect on Ovine Red Cells	J. W. Eaton, E. Berger, D. Nelson, J. G. White, O. Rundquist	506

IMMUNOLOGY

Heterogeneity of KCl-solubilized Antigens of Chemically Induced Sarcomas	R. Blackstock, R. D. Schimpff, R. T. Smith	354
Preferential Depression IgM Producing Immunocytes during Mastocytoma-induced Immunosuppression	H. Friedman, I. Kamo	381
Stimulation of Immune Response in Hybrid Mice Following Rauscher Virus Infection	J. P. Okunewick, R. F. Meredith, B. Brozovich, E. V. Weaver	449
Enhanced Expression of Histocompatibility Antigens on Interferon-Treated Mouse Embryonic Fibroblasts	F. Vignaux, I. Gresser	456

Correlation of Chromium Sesquioxide and [^{14}C]Cellulose as Fecal Markers in Rats — L. P. Foreman, B. O. Schneeman, W. C. Weir 418
Cellular Antibody Synthesis in Thiamin, Riboflavin, Biotin and Folic Acid-Deficient Rats — M. Kumar, A. E. Axelrod 421
Regulation of Food Intake During Long-Term Loss of Food from the Intestines of the Rat — H. S. Koopmans 430

ONCOLOGY

Granulocyte Colony Formation in Chronic Granulocytic Leukemia during Stable, Accelerated and Blastic Disease — C. A. Rhodes, W. A. Robinson, M. A. Entringer 337
Levels of Blood Group Synthetic Enzymes in Human Colonic Carcinoma — D. T. Kiang, B. J. Kennedy, R. D. Edstrom 411
Increased Lethality after Endotoxin in Old or Leukemic AKR Mice — S. S. Boggs, G. N. Schwartz 424
Effect of the Prolonged Immunization with Tumor-Unrelated Antigen on the Tumor Growth — S. P. Targowski, B. Albini, F. Milgrom 511
Growth Promoting and Inhibitory Activities of 3T3 and Other Cell Lines for Thiol-dependent Lymphoma Cells *in Vitro* — P. Tanapat, E. Gaetjens, J. D. Broome 517

PHARMACOLOGY

True and False Prophylaxis in Experimental Trypanosomiasis — N. Ercoli 397
Effect of Antipyretics on group A Streptococcal Pyrogenic Exotoxin Fever Production and Ability to Enhance Lethal Endotoxin Shock — P. M. Schlievert, K. M. Bettin, D. W. Watson 472

PHYSIOLOGY

Effects of Acute Fluoride Intoxication on Rats — L. Singer, W. D. Armstrong, R. H. Ophaug 363
Bile Acids in Bile of Germfree and Conventional Dogs — M. H. Beaver, B. S. Wostmann, D. C. Madsen 386
The Distribution of Blood Flow Along the Small Intestine of the Dog — M. N. Eade, R. W. Ginn 390
Independent Effect of Bicarbonate on Renal Citrate Metabolism — D. S. Fraley, S. Adler 393
Stimulation of Gastrin Release in Dogs by Individual Amino Acids — U. T. Strunz, J. H. Walsh, M. I. Grossman 440
Effect of Anionic and Nonionic Contrast Media on Renal Extraction of Para-Aminohippurate in the Dog — G. F. DiBona 453
Release of Tissue Taurine from the Oxygen-Deficient Perfused Rat Heart — M. F. Crass, III, J. B. Lombardini 486

RADIOBIOLOGY

Long-Term Adjuvant Effect of Bacterial Endotoxin in Prevention and Restoration of Radiation-Caused Immunosuppression — U. H. Behling, A. Nowotny 348

TISSUE CULTURE

Squamous Metaplasia of the Tracheal Epithelium in Organ Culture. II. Nutritional Influences — B. T. Mossman, N. Heintz, B. V. MacPherson, J. E. Craighead 500

VIROLOGY

Circulating Interferon in Sudden Infant Death Syndrome	D. S. Y. SETO, D. H. CARVER	378
A Cardiomyopathy of BALB/c Mice (Superimposed Infection by Coxsackievirus B-3)	F. M. WILSON, A. M. LERNER	442
Properties of Epstein-Barr Virus Transformed Woolly Monkey Lymphoblastoid Cell Lines	W. A. ANDIMAN, G. MILLER	489

; OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 337-341 (1978)

ınulocyte Colony Formation in Chronic Granulocytic Leukemia during Stable, Accelerated and Blastic Disease (40049)

C. A. RHODES, W. A. ROBINSON, M. A. ENTRINGER

Department of Medicine, University of Colorado Medical Center, Denver, Colorado 80262

ıies of maturing granulocytes and hages can be grown from the periph- od and bone marrow of normal hu- ings as well as from patients with a riety of hematopoietic disorders (1–4). ɛ granulocytic leukemia little or no formation takes place from leukemic 6). A number of workers have shown, r, that in chronic granulocytic leuke- ɜL), at least in the stable phase of the there are increased numbers of gran- colony forming cells (CFU-C) circu- ı the peripheral blood and the bone (3, 5, 7, 8). It has also been suggested number of such colony forming cells ɛs in the bone marrow as the acceler- blast phase of this disease ensues (3, data supporting this contention has, r, been scanty.

present studies were undertaken to ne the number of CFU-C in the pe- blood and bone marrow of patients GL during various phases of their These studies have corroborated pre- ɾork indicating that the number of in peripheral blood during the stable f the disease is high. Unlike previous however, the present work has shown the accelerated and blast phase of .at colony forming cell numbers may high or very low, but are not consist- ɛcreased.

rials and methods. All patients in this ere seen at the University of Colorado l Center between January 1, 1970 and 1977. The diagnosis of CGL was pon a hypercellular bone marrow, the ɛ of a low leukocyte alkaline phos- score and/or the presence of the Phil- ı chromosome in bone marrow peripheral blood preparations. All of udies were performed after written d consent as approved by the Human h Committee of the University of o Medical Center. The definitions of various stages of the disease as used in this study are as follows:

Stable phase. Patients were placed in the stable phase if they were either off all medication or on maintenance therapy and had hematocrits above 30, platelet counts above 100,000 and white blood cell counts (WBC) less than 50,000 with less than 5% blasts and promyelocytes. Three patients whose WBC counts cycle periodically above 50,000 were included because they appeared clinically to be in the stable phase of their disease.

Accelerated phase. The criteria of Schwartz and Canellos were used to define the accelerated phase (9). Patients who exhibited a gradual acceleration in their disease with progressive anemia, thrombocytopenia, leukocytosis, splenomegaly, or thrombocytosis unresponsive to previously effective therapy were considered to be in accelerated phase. Leukocytosis with up to 20% blasts was felt consistent with accelerated phase, over 20% with blast phase.

Blast phase. The criteria for definition of blast phase were drawn from previous work by Schwartz and Canellos (9), Canellos *et al.* (10), and Karanas and Silver (11). Patients who had a rapid acceleration of their disease with leukocytosis greater than 30,000/mm^3, anemia with a hemoglobin less than 10 gm%, and thrombocytopenia with platelets less than 100,000, with an FUO with T greater than 38.5° for 5 days; or with an increasing percentage of blasts in the marrow (20–30%) were considered to be in blast crisis. Patients with leukocytosis and greater than 20% blasts in the peripheral blood were placed in the blast phase category.

Fortunately, the designation of patients into the above categories was readily apparent. No difficulty was encountered in separating those in the accelerated phase from those in the blast phase.

Preparation of peripheral blood and bone marrow cells for colony growth. The tech-

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nique used here was that of Pike and Robinson using a double layer agar system (1). The stimulus for colony formation in all of these studies was peripheral white blood cell feeder layers derived from the peripheral blood of normal human beings. These were prepared as previously described in 0.5% agar in McCoy's 5A medium with 15% fetal calf serum in a concentration of 1×10^6 cells/ml, and stored in a fully humidified CO_2 incubator at 37° for up to 14 days prior to use.

Peripheral blood from patients was collected in heparinized vacuum tubes, allowed to sediment by gravity at room temperature and the white blood cell rich plasma removed. Peripheral white blood cells were then counted and plated in at least triplicate plates at concentrations of 1×10^5 and 2×10^5 nucleated cells per ml in 1 ml overlayers in 0.3% agar.

Bone marrow was collected by aspiration of the posterior iliac crest in heparinized syringes. This was followed by sedimentation at gravity at room temperature for 1–2 hr at which time the buffy coat plasma was withdrawn, the nucleated cells counted and plated as above at concentrations of 1×10^5 and 2×10^5 cells/ml. In the present studies no attempts were made to remove colony stimulating cells from the bone marrow or peripheral blood overlays.

After preparation the plates were allowed to gel at room temperature and then incubated in a fully humidified incubator at 37° for 14–18 days at which time the colony counts were performed with the aid of a dissecting microscope. Only colonies containing 50 or more cells were counted.

Results. Figure 1 shows the number of CFU-C found in the peripheral blood of patients with CGL during the stable, accelerated, and blast phases of the disease. In the stable phase of the disease 43 determinations were made on 16 patients. The mean number of CFU-C per ml of blood was 107×10^3. This is almost twice the value found in normal human peripheral blood which in our laboratory ranges from 0 to 50×10^3 colonies per ml (2). Note particularly that the number of colony forming cells in peripheral blood has been expressed per ml of blood rather than per number of cells plated. This is important because of the extreme variability in

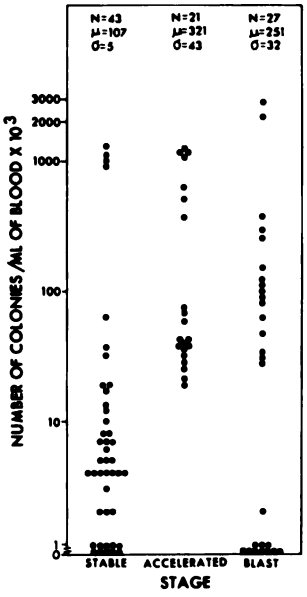
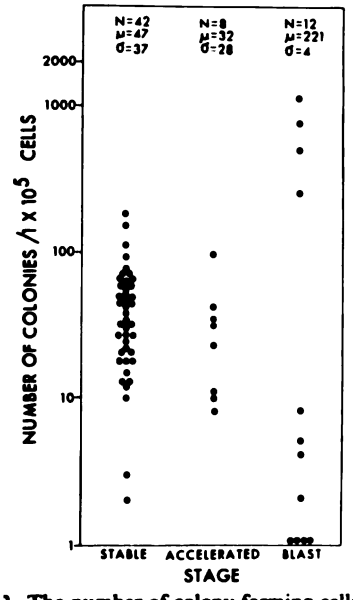


FIG. 1. The number of colony forming cells per ml of peripheral blood in patients with chronic granulocytic leukemia during the stable, accelerated, and blast phases of the disease. Each point is the mean colony count of data from one patient. The number of determinations (η), mean (μ), and median (σ) values are given.

the peripheral white blood cell numbers in these patients. In the accelerated phase of the disease, the number of colonies was further increased to 6 times the normal value with a mean colony count of 321×10^3/ml. The scatter was, however, quite broad ranging from 19×10^3/ml to 1260×10^3/ml. The data shown in Fig. 1 represents 21 data points obtained on 10 patients using the criteria for accelerated phase disease as outlined earlier. The wide scatter noted suggests that the median determination may be a more important value. This is also shown in Figure 1 for all phases of CGL and indicates that when the number found in the stable phase of the disease (5×10^3) is compared to the accelerated phase an increase (43×10^3/ml of blood) is also found. During the blast phase of the disease the number of colony forming cells was again increased over that found in normal humans and in the stable phase of CGL with a mean of 251×10^3/ml as shown in Fig. 1. The data shown here represents 27 data points on 11 separate patients. Here, once again, the scatter was quite broad. Determination of the median shown in Fig. 1,

ed that the value was higher (32 × an that seen in the chronic phase of ease but similar to that found in the ated phase. Attempts to correlate the r of CFU-C in the peripheral blood bsolute white blood cell counts, the s response to therapy or subsequent outcome were unsuccessful. Those s with high colony forming cell num- the accelerated or blast phase of the did not differ significantly clinically ıose who had low or decreased colony g cell numbers.

re 2 shows the number of CFU-C in ıe marrow of the same patients during ; phases of their disease. Unfortu- in the accelerated and blast phase of ease bone marrow aspirates are often ible to obtain and the number of data available was somewhat less. In these the number of colonies has been ex- l as per 1 × 10^5 cells plated. In the phase of the disease 42 data points ɔtained on 22 patients. The mean num- ɔolonies grown was 47 and the median



2. The number of colony forming cells per 1 × plated in the bone marrow of patients with granulocytic leukemia during the stable, accel- nd blast phases of the disease. Each point is the ony count of data from one patient. The number ninations (η), mean (μ), and median (σ) values n.

was 37. The mean number of colonies during the stable phase of the disease is surprisingly not significantly higher than we have recently reported for normal human bone marrow grown under the same conditions (12). In those studies, 99 normal human bone marrows were plated at 2 × 10^5 cells and the mean colony forming cell number was closely clustered around 75 with a range from 24 to 250. It is unlike data reported by previous authors which indicated that the number of CFU-C in the bone marrow was markedly increased in stable CGL (3–7). Also shown in Fig. 2 are the number of CFU-C in the bone marrow of patients during the accelerated phase of the disease. Shown here are eight data points obtained on four patients. The number of CFU-C is reduced compared to normal values with a mean of 32 and a median of 28. In contrast, the mean number of CFU-C in the bone marrow of patients during the blast phase of their disease is markedly elevated at 221 colonies/10^5 cells plated. The data shown here contain 12 data points obtained on six patients. It can be seen that there was considerable scatter and when the median colony count, as opposed to the mean, is determined, this value falls to 4 CFU-C per 1 × 10^5 cells. It would appear from the data shown in Fig. 2 that in the blast phase of CGL there are two separate populations of patients, one group with high numbers of CFU-C in the marrow and another group with low numbers. No clinical differentiation between these patients could be determined, however.

There was a direct correlation between the numbers of CFU-C found in the peripheral blood and bone marrow during all phases of the disease. When high numbers of CFU-C were present in the blood, high numbers were also found in the bone marrow. The reverse was also found and is illustrated by the four patients with very low or absent bone marrow CFU-C during the blastic phase. In all four concomitant determinations of peripheral blood CFU-C also showed that they were low or absent.

Colony morphology of peripheral blood and bone marrow colonies was studied using Wright-Giemsa stained preparations. No definite differences in cellular maturation of colony cells were noted when compared between

groups or with normal human bone marrow colonies as described previously (1).

Discussion. These data have confirmed previous reports that the number of granulocyte colony forming cells in the peripheral blood of patients with chronic granulocytic leukemia is increased during the chronic or stable phase of the disease. These data have not confirmed previous reports that the number of CFU-C in the bone marrow is increased, however, nor have they shown a consistent decrease in the colony forming cell numbers in the peripheral blood or bone marrow with the advent of blast crisis, a claim which has been put forward previously (3, 4).

The reasons for the differences in colony growth noted between previous reports and those reported here do not apparently lie in the heterogeneity of patients studied. Clinical details for the patient group studied here have not been presented but they all represent so-called "classic" chronic granulocytic leukemia with the presence of the Philadelphia chromosome and/or low leukocyte alkaline phosphatase scores. Likewise, their clinical outcome with relationship to longevity or response to therapy cannot be equated with the various colony forming cell numbers noted.

It is likely that these results represent the biologic variability that might be expected in such a disease and that this represents the first large study specifically related to this disorder.

Perhaps of interest to the present studies has been the recent finding of the presence of terminal nucleotidal transferase on the cells undergoing blast crisis in chronic granulocytic leukemia (13). This data has suggested that there may be progressive lymphocyte involvement in this disorder with the final development of acute lymphoblastic leukemia. Previous studies from this laboratory have shown that the number of CFU-C in bone marrow of children with acute lymphoblastic leukemia is markedly reduced compared to normal humans (14). It would be of extreme interest to determine whether those patients in the present studies who had reduced numbers of CFU-C in the peripheral blood and bone marrow during the advanced stages of their disease were also those who appear to transform to a lymphocytic form of leukemia. These studies have not been performed to date but are currently being considered.

We originally hoped that determination of colony forming cell numbers in peripheral blood and bone marrow might be of prognostic importance and significance. The initial suggestion that as blast crisis ensued the number of colony forming cells decreased led us to consider the possibility that we might use this indicator as a guide to therapy, particularly early intervention with more aggressive chemotherapy before histologic evidence of blast crisis occurred. The present studies indicate that such an approach is neither feasible nor warranted. Patients with clear-cut blast crisis retain large numbers of colony forming cells in their peripheral blood and bone marrow. In our hands this appears to occur in about half the patients. It is possible that with serial points on a single patient one might be able to postulate the eventual outcome of the disease but this is probably not more beneficial than simple histologic viewing of changes in the morphology of the peripheral blood and bone marrow cells.

The present findings, when taken in the light of other recent studies in CGL suggest a considerable biologic and pathophysiologic spectrum in this seemingly uniform disorder of human beings.

Summary. The number of granulocyte colony forming cells (CFU-C) has been determined in the peripheral blood and bone marrow of patients with chronic granulocytic leukemia (CGL) during the stable, accelerated and blastic phases of the disease. These studies have corroborated previous work indicating that blood CFU-C numbers are high during the stable phase of CGL. In the accelerated and blast phases of CGL, CFU-C numbers in the peripheral blood and bone marrow may be decreased, normal or increased, but are not consistently low. Changes in CFU-C numbers could not be correlated with clinical patterns of CGL, or eventual outcome of the disease.

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Biochemical Properties of Bovine Granulocytes (40050)

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A number of investigations carried out *in vitro* on isolated granulocytes have allowed an increase in knowledge on the molecular mechanisms of host defense against invading microorganisms (1, 2). In addition, these investigations have contributed to a better understanding of cell events of broader biological interest, such as those concerning signal transduction from the cell environment to the cytosol, activation of metabolic pathways, modulation of cell functions by cytoskeletal components, secretion of granule associated enzymes (3, 4).

For some of these studies, in particular those involving separation of either subcellular fractions or enzymes, fairly large amounts of cells are required. For this purpose, granulocytes isolated from exudates of laboratory animals are generally used. So far most of the biochemical studies on blood derived granulocytes have been performed on cells of human blood, either donated by volunteers or supplied by blood banks. This sometimes might cause limitations because of the amount of cells available. We, therefore, thought it might be useful to secure another source of blood granulocytes with a precise biochemical characterization. In this paper we describe the ultrastructure, enzyme content and features of the oxidative metabolism of granulocytes isolated from bovine blood, which can be obtained in unlimited amounts from slaughterhouses, and compare the biochemical properties of these cells with those of granulocytes of human blood.

Materials and methods. Granulocytes were isolated from bovine blood, essentially as described by Carlson and Kaneko (5), washed once and suspended in Ca^{2+}-free Krebs–Ringer phosphate (KRP), pH 7.4. The white cell fraction of human blood anticoagulated in ACD was isolated by a conventional technique, after dextran sedimentation of the erythrocytes. Differential counts were performed on May-Grünwald-Giemsa stained smears.

Protein was determined by the method of Lowry *et al.* (6), using bovine serum albumin (BSA) as standard. Myeloperoxidase was measured biochemically as described by Romeo *et al.* (7) and histochemically according to Graham and Karnovsky (8). β-Glucuronidase was determined fluorimetrically (9) in 0.5 ml of 0.05 *M* Na-acetate buffer, pH 3.8, containing 0.05% BSA with 0.32 m*M* 4-methylumbelliferyl-β-D-glucuronide (Sigma, USA) as substrate; the reaction was stopped with 1.5 ml of 0.2 *M* Na-glycine buffer, pH 10.45. β-Galactosidase was assayed by the same procedure except for the pH of the buffer, which was 4.5, the substrate being 0.54 m*M* 4-methylumbelliferyl-β-D-galactoside (Sigma, USA). Acid phosphatase was measured in 0.5 ml of 0.1 *M* Na-acetate buffer, pH 4.5, with 4.5 m*M* *p*-nitrophenyl phosphate (Sigma, USA) as substrate (10); the reaction was stopped with 0.5 ml of 1 *M* NaOH. The rate of splitting of 4.5 m*M* *p*-nitrophenyl phosphate by alkaline phosphatase was directly recorded at 410 nm in 1 ml of 0.1 *M* diethanolamine-HCl buffer, pH 9.7, with 1 m*M* $MgCl_2$. Lysozyme was determined nephelometrically by the rate of lysis of cell walls of *Micrococcus lysodeikticus* in Na-phosphate, pH 6.6 (ionic strenght = 0.5); crystalline hen egg-white lysozyme (S.P.A., Italy) was used as standard. The oxidation of NADH by oxalacetate was followed to measure the activity of malate dehydrogenase (11). Catalase was assayed by the rate of decrease of absorbance of H_2O_2 at 230 nm (ϵ_{230} = 0.067 cm^{-1} mM^{-1}) (12). The activity of glutathione peroxidase and glutathione reductase was determined at 37° in a final volume of 1 ml of 20 m*M* KH_2PO_4-Na_2HPO_4 buffer, pH 7.0, essentially as described by Reed (13). For assay of glutathione peroxidase, cuvettes contained, in addition to the above, 5 m*M* ethylenediaminotetraacetate (pH 7.0), 1 m*M* sodium azide, 0.01% BSA, 1 m*M* GSH, 0.1 m*M* NADPH, 30 mU of yeast glutathione reductase (Boehringer, Germany)

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.1 m*M* H_2O_2. For assays of glutathione tase, cuvettes contained 0.01% BSA, 1 GSSG and 0.1 m*M* NADPH.

e determination of the activity of catalglutathione peroxidase and glutathione tase was carried out with postgranular natants, obtained by centrifugation 0*g*, 10 min) of cells disrupted by honization in 0.34 *M* sucrose (7). All the assays were performed with cells dis-d by sonication (Branson Sonifier, 3.5 10 sec). The reaction media of β-gluidase, β-galactosidase, acid and alkaline phatase, and malate dehydrogenase also ined 0.05% Triton X-100. All the enassays were shown to be linear with t to time of incubation and amount of xtracts or sonicates added to the me-. The rates of reactions were corrected nenzymatic rate before the calculation cific activity of enzymes.

amin B_{12}-binding capacity of cell soniwas measured by the method described ane *et al.* (14). The incubation mixture) contained 0.55 ng of ^{57}Co-B_{12} (0.18 g, Radiochemical Centre, England) and cold vitamin B_{12}; excess free vitamin as removed with 0.5 ml of albumin-d charcoal and 1 ml of the centrifuged les was counted.

imation of lactoferrin content of bovine locytes was carried out by Ouchterlony e immunodiffusion in agarose, and ng of the immune precipitates was per-d with amido black according to Wieme Cells were extracted either in KRP (pH ontaining 0.3% cetyl trimethyl ammobromide (16) or in 1 *M* NaCl overnight (17) (an identical recovery of lactoferrin btained with the two extraction proce-). The extracts were reacted with anti- to bovine milk lactoferrin (a gift of P. Masson), and purified bovine lacto- (a gift of Prof. B. Senft) was used as ard antigen. Twelve dilutions of the ard antigen (from 1 to 100 μg/ml) and st six dilutions of granulocyte extracts 1 to 10 × 10^6 cells/ml) were tested st the antiserum.

gocytic stimulation of granulocyte mesm was accomplished by exposing the at 37° to autoclaved *Bacillus mycoides*, ized with fresh bovine serum. Oxygen consumption, superoxide anion recovery, hydrogen peroxide accumulation and ^{14}C-glucose oxidation were measured as described by Dri *et al.* (18).

Escherichia coli J53 (K 12) and *Staphylococcus aureus* (strain Oxford) were grown overnight in nutrient broth and BHI respectively, washed once and suspended in KRP, pH 6.0 or pH 7.4, with 0.5 m*M* $CaCl_2$. Bactericidal activity was determined by adding 1 × 10^7 bacteria to 2 × 10^6 granulocytes disrupted by sonication, frozen at −70° and then thawed, in a final volume of 0.3 ml of KRP, pH 6.0 or pH 7.4, with 0.5 m*M* $CaCl_2$. After 30 min incubation at 37°, aliquots were diluted and plated to obtain viable cell counts. Controls were run in the absence of either bacteria or granulocyte sonicates.

For electron microscopic observations cells were fixed at 4° for 60 min in 2% glutaraldehyde (0.1 *M* cacodylate buffer, pH 7.4), postfixed in 1% osmium tetroxide, dehydrated in ethanol, carried through propylene oxide, embedded in DER 332, and counterstained with uranyl acetate and lead citrate.

Results. We carried out thirty cell preparations with an average yield (± SEM) of 1.25 (± 0.03) × 10^6 white cells per ml of bovine blood. Of these cells 96.7 (± 0.1)% were granulocytes, the ratio between neutrophils and eosinophils varying from 200:1 to 5:1. By processing two or more blood samples at the same time, cell preparations with the highest content of eosinophils could be discarded. By following this procedure we were able to perform the biochemical assays on cell suspensions containing an average of 92% neutrophils (see Table I).

The electron microscope observation of bovine blood granulocytes (Fig. 1) shows that neutrophils are relatively small (diameter about 7 μm), rounded or slightly elongated cells with occasional short cytoplasmic projections. Several spherical, elongated, rod and dumb-bell shaped granules, are noticed in the dense cytoplasm. They are all highly and homogeneously electron-dense. In many cell sections a roughly circular, granule-free area is observed in the cytoplasm, close to the nuclear lobes, but sometimes in an eccentric position. This is the centrosphere region, circumscribed by stacks of four to nine smooth-surfaced, curved Golgi cisternae, oriented

TABLE I. ENZYME ACTIVITIES AND CONTENT OF BINDING PROTEINS OF GRANULOCYTES OF BOVINE AND HUMAN BLOOD.[a]

	Bovine granulocytes	Human granulocytes
1. β-Glucuronidase	12.8 ± 0.3 (8)	202.1 ± 18.0 (3)
2. β-Galactosidase	11.8 ± 0.6 (9)	62.4 ± 7.4 (3)
3. Myeloperoxidase	1.6 ± 0.1 (8)	4.7 ± 0.1 (3)
4. Acid phosphatase	36.7 ± 6.3 (5)	—
5. Alkaline phosphatase	230 ± 9 (8)	23.9 ± 7.0 (3)
6. Lysozyme	< 0.2 (3)	31.8 ± 3.6 (3)
7. Lactoferrin	74 ± 7 (7)	30–64 (Refs. 16, 33)
8. Vitamine B_{12}-binding capacity	26.7 ± 2.8 (3)	4–9 (Refs. 34–36)
9. Malate dehydrogenase	0.49 ± 0.04 (6)	—
10. Catalase	7.1 ± 0.5 (3)	108.2 ± 11.1 (4)
11. Glutathione peroxidase	14.1 ± 0.6 (4)	5.2 ± 0.4 (4)
12. Glutathione reductase	25.0 ± 0.8 (4)	13.2 ± 1.1 (4)
13. Protein	1.21 ± 0.03 (14)	—

[a] Sixteen preparations of bovine blood granulocytes and four preparations of human blood granulocytes were used for the assays, which were run as described in the section on Materials and Methods. The bovine cell preparations had the following average % composition (±SEM): neutrophils 92.1 ± 0.4; eosinophils 5.3 ± 0.3; monocytes 2.6 ± 0.1. Data are expressed as means ±SEM/10^7 cells; the number of experiments is shown in brackets 1 and 2: nmoles methylumbelliferone/30 min; 3: μmoles *t*-guaiacol/min; 4 and 5: nmoles *p*-nitro phenol/min; 6 and 7: μg; 8: ng vit B_{12}; 9: μmoles NADH/min; 10: μmoles H_2O_2/min; 11 and 12: nmoles NADPH/min; 13: mg.

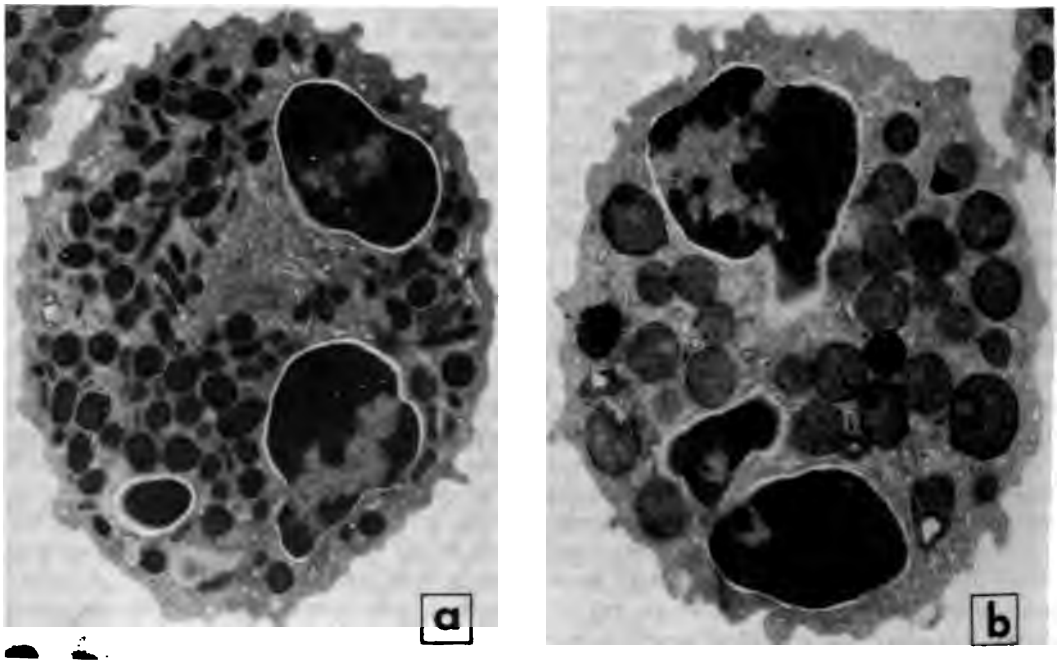

FIG. 1. Ultrastructure of a bovine blood neutrophil (a) and of a bovine blood eosinophil (b) (× 12,600).

around one or two centrioles. The cytoplasm also contains particulate glycogen and a few vesicular profiles of rough-surfaced endoplasmic reticulum but lacks appreciable quantities of most other organelles (e.g. mitochondria, ribosomes, microtubules).

Figure 1 also shows that bovine blood eosinophils have much larger granules than the neutrophils, as reported also by others (19). These granules lack the crystalloid structure characteristic for eosinophil granules in other species (20). In this regard, bovine eosinophils resemble eosinophils of horse (21), of mink (22) and of some domestic birds (23).

In Table I bovine granulocytes are compared with human leucocytes on the basis of

s and/or content of a number of en-
ınd binding proteins, selected on the
either their potential use as markers
ellular fractions or their involvement
xidative metabolism.

ıctivities of β-glucuronidase, β-galac-
and myeloperoxidase, which in other
are associated to azurophilic granules
25), are much lower in the bovine
the human granulocytes. A histo-
ıl survey on bovine granulocytes in-
that myeloperoxidase is particularly
rated in eosinophils (data not shown),
ggesting that the value of myeloperox-
ctivity in the preparations of bovine
hils might in part depend on the con-
ion by eosinophils.

concentration of lactoferrin and vi-
$_{12}$-binding protein, markers of specific
s in other species (17, 25, 26), appears
gher in the bovine than in the human
cytes. As reported also by others (27),
ıe activity is undetectable in bovine
cytes.

respect to the human leucocytes, the
granulocytes have a very low activity
ase, the enzyme which provides an
system for H_2O_2 detoxification dur-
gocytosis (12). This deficiency, how-
counterbalanced by a higher specific
of the two enzymes of the glutathione
3), glutathione peroxidase and gluta-
eductase.

exposure of granulocytes to bacteria
marked stimulation of oxygen con-
n, O_2^- and H_2O_2 generation, and glu-
idation through the hexose mono-
ıte pathway (HMP) (3, 13, 18, 28–30).
luation of the metabolic changes oc-
curring in phagocytizing bovine leucocytes, and of the modulation exerted on them by the heme-enzyme inhibitor NaN_3 and by the O_2^- oxidizing agent ferricytochrome *c*, is reported in Table II. The significance of these data will be discussed below.

Finally, the oxygen-independent bactericidal activity of bovine granulocytes, as compared with the activity of human leucocytes, is shown in Table III. With *E. coli* the cidal efficiency of the two types of phagocytes is very similar, the activity of the lysates of bovine granulocytes being somewhat lower than that of human cell lysates at pH 7.4. On the contrary, at both pH's killing of *S. aureus* is carried out only by the bovine cell lysates.

Discussion. In this paper we have reported several biochemical features of preparations of bovine granulocytes containing an average of 92% neutrophils and less than 3% monocytes. Some enzyme properties of granules of bovine granulocytes have been already described by Hegner (19) and by Rausch and Moore (27). At least in the former case, however, cell preparations were heavily contaminated by mononuclear cells and had a high eosinophil/neutrophil ratio.

When compared to human granulocytes, the bovine cells appear to exhibit low activities of enzymes, which in other species are associated to azurophil granules (10, 24, 25), and to have a higher content of binding proteins, which belong to specific granules (17, 25, 26). Whether this is due to a different cell content of these two types of granules is difficult to assess on a morphological basis, since in bovine granulocytes azurophil and specific granules cannot be distinguished on the basis of electron density (31).

II. STIMULATION OF THE OXIDATIVE METABOLISM OF BOVINE GRANULOCYTES BY PHAGOCYTOSIS.[a]

	Resting granulocytes	Granulocytes + *B. mycoides*			
		—	+NaN_3	+cyt *c*	+cyt *c* +SOD
ı/2 min	8.9 ± 1.4	77.9 ± 6.7	87.6 ± 8.3	70.1 ± 5.6	77.9 ± 8.2
ı$^-$/2 min	—	—	—	26.1 ± 6.1	—
$_2O_2$/2 min	—	8.6 ± 1.3	27.1 ± 3.3	—	—
n/μCi 1-[^{14}C]Glc/10 min	6,665	100,525	—	—	—
n/μCi 6-[^{14}C]Glc/10 min	110	190	—	—	—

of O_2 consumption, O_2^- recovery and H_2O_2 accumulation are means of 4 experiments ±SEM (stimulated ıs are increments with respect to resting controls); a detailed description of the experimental conditions for these measurements is given in Ref. 18. Rates of glucose oxidation are results of a representative ıt. SOD: superoxide dismutase.

TABLE III. COMPARATIVE BACTERICIDAL ACTIVITY OF LYSATES OF BOVINE AND HUMAN BLOOD GRANULOCYTES.[a]

	E. coli		*S. aureus*	
	pH 6.0	pH 7.4	pH 6.0	pH 7.4
Bovine granulocyte lysates	66%	59%	13%	65%
Human granulocyte lysates	63%	85%	0	0

[a] 1×10^7 bacteria were incubated at 37° for 30 min with lysates of 2×10^6 granulocytes in 0.3 ml of KRP, pH 6.0 or 7.4, containing 0.5 m*M* $CaCl_2$. Data are average percentages of killing, calculated with respect to viable bacteria found at the end of incubations run in the absence of granulocyte lysates (two to three experiments).

The granules of bovine granulocytes appear to be endowed with bactericidal substances capable of killing *S. aureus* under conditions in which this microorganism is not killed by human leucocytes. The cidal activity of the bovine cell lysates on the gram-positive bacterium is more marked at pH 7.4 than at pH 6.0, thus suggesting that granule components with optimal activity near or above neutrality (32) are mainly responsible for the toxic effects on the staphylococci.

The exposure of bovine granulocytes to bacteria causes a stimulation of their oxidative metabolism, which is similar to that known to occur in human and guinea pig granulocytes (3, 13, 18, 28–30). The main product of the enhanced oxygen reduction in phagocytizing cells is likely to be O_2^-, which rapidly generates H_2O_2 by dismutation (18). The decrease of oxygen consumption by phagocytizing granulocytes observed in the presence of ferricytochrome *c* is due to the oxidation of the extracellularly released O_2^- to O_2. From the extent of the superoxide dismutase-inhibitable reduction of ferricytochrome *c* and on account of the stoichiometries of the O_2^- dismutation and of the catalatic degration of H_2O_2 (18), the amount of O_2^- released by phagocytizing bovine granulocytes under our experimental conditions can be calculated to be about 15%. This recovery is comparable to that obtained with human granulocytes under the same conditions (unpublished experiments).

The main mechanisms of H_2O_2 disposal in phagocytizing granulocytes involve catalase, myeloperoxidase and glutathione peroxidase (12, 13, 18, 28). By inhibiting the catalase-dependent back-conversion of H_2O_2 to O_2, NaN_3 enhances the overall O_2 consumption (18). In bovine granulocytes this enhancement is about fourfold lower than in human leucocytes (unpublished experiments) due to the deficient activity of catalase. The inhibition of catalase and myeloperoxidase by NaN_3 also causes an increased accumulation of H_2O_2, which in guinea pig granulocytes approaches 100% of the value expected from oxygen reduction (18). On the contrary, in phagocytizing bovine granulocytes, the average value of H_2O_2 measured in the presence of NaN_3 is only a small percentage of the amount calculated from the values of oxygen consumption. This suggests that in these phagocytes H_2O_2 is largely utilized by the NaN_3-insensitive glutathione cycle (13). This conclusion is consistent with (i) the low activity of catalase; (ii) the relatively high activity of glutathione peroxidase and glutathione reductase; and (iii) the remarkable activation of the HMP by phagocytosis, which is higher than that reported for granulocytes of man and guinea pig (28). The stimulation of the glutathione cycle leads, in fact, to increased generation of $NADP^+$ with activation of HMP (13).

In conclusion, bovine blood granulocytes, apart from the absence of lysozyme and from the different metabolic fate of H_2O_2, have biochemical properties very similar to those of human granulocytes. Since they can be obtained in unlimited amounts from slaughtered animals, bovine granulocytes can substitute for human cells in laboratory investigations, in particular when the isolation of large quantities of subcellular particles, enzymes and bactericidal factors is required.

Summary. Granulocyte fractions, containing an average of about 92% neutrophils, were isolated from bovine blood. The electron microscope observation of these fractions showed that neutrophil granules have different shapes, but are all highly and homogeneously electron-dense. With respect to the granulocytes of human blood, bovine cells appear to have a lower content of azurophil enzymes and virtually lack lysozyme. Lysates of bovine granulocytes efficiently kill both *E. coli* (at pH 6.0 and 7.4) and *S. aureus* (mainly at pH 7.4). When exposed to opsonized *B*

mycoides, intact bovine granulocytes exhibit a marked enhancement in oxygen consumption, generation of O_2^- and H_2O_2, and glucose oxidation through the hexose monophosphate pathway. About 15% of the total oxygen reduced is recovered extracellularly as O_2^-. Hydrogen peroxide generated by phagocytizing cells is only partially utilized in reactions catalyzed by catalase and myeloperoxidase, and appears to mainly enter the glutathione cycle.

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Long-Term Adjuvant Effect of Bacterial Endotoxin in Prevention and Restoration of Radiation-Caused Immunosuppression (40051)

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One of the first observations of the effects of whole-body irradiation on the immune system was the resultant impaired resistance to infections evidenced by death of the animal or by quantitative measurements of the immune response to a specific antigen. Benjamin and Sluka (1) observed that exposure to x-rays resulted in the loss of antibody formation. Since that time, an overwhelming body of literature on the effects of irradiation on infections has been documented, including a large number of reviews (2–4). There is little doubt that the major cause of increased susceptibility to infection in irradiated animals exhibiting the "acute radiation syndrome" can be attributed to the loss of function of the immune system (5).

The protective effect of endotoxin against lethal irradiation was described first by Mefferd *et al.* (6) and studied in detail by others, with particular emphasis on the effect of endotoxin on hemopoiesis (7, 8). The adjuvant property of endotoxin was described first by Johnson *et al.* (9). Kind and Johnson (10) applied endotoxin simultaneously or one or two days following antigenic stimulation and observed good adjuvant effect to bovine γ globulin in rabbits. No adjuvant effect was obtained if the endotoxin was given before the antigen. Similar results were obtained by Franzl and McMaster (11), injecting mice with sheep erythrocytes following relatively high doses of endotoxin. Kind and Johnson (10) also attempted to prevent radiation-caused immunosuppression. If the antigen was given together with endotoxin, the immune response was about 50% of that elicited by antigen alone in normal animals.

Our earlier studies in this field were based on the assumption that the stimulatory effects of endotoxin on hemopoiesis and immune functions play a vital role in the protective effects of endotoxin in lethally irradiated animals. We found that the radioprotective effect was strongly time-dependent. The protection showed a biphasic profile when single low doses of endotoxic lipopolysaccharide (LPS) or of a polysaccharide-containing (PS), nontoxic fraction of LPS were administered prior to irradiation. Very recently we reported alternating immunosuppressive or immunopotentiating effects of LPS and PS, depending upon the time of their application relative to the time of immunization (12, 13). The aims in our present study were to use optimal treatment times for the adjuvant effect of endotoxin and thereby to attempt to prevent or repair the irradiation-caused immunosuppression by such endotoxin treatment.

Materials and methods. Experimental animals. All mice used were 10–14 week-old females weighing between 18–25 g. ICR mice were obtained from the Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania.

Endotoxic lipopolysaccharide (LPS). Lipid-extracted *Serratia marcescens* 08.00 cells were homogenized with 5% trichloroacetic acid (TCA). The extracted crude antigen was precipitated in cold methanol and purified by ultracentrifugation. The sediment was redissolved in deionized water, dialyzed and lyophilized.

Antigens. Sheep red blood cells (SRBC) were obtained commercially in a 25% by volume Alsever's solution from Rockland Farms, Gilbertsville, PA. The cells were washed and centrifuged three times with sterile saline, counted and adjusted to the proper cell concentration.

Radiation. The radiation source was a General Electric Maxitron 300 x-ray machine operated at 300 Kvp, 20 ma, with added filtration of 0.26 mm Cu and 1.04 mm Al, yielding a half-value layer of 1.10 mm Cu. At a source-to-target distance of 60 cm, the air exposure dose rate was between 230 and 240 r/min, as measured by a Victoreen air ionization chamber model 154 which was corrected for temperature and pressure variations.

Rosette-forming cell (RFC) assay. Our

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l involved a modified technique first ed by Zaalberg (14). Mice in groups were injected ip with 10 μg LPS either ıneously with SRBC or at designated tervals, and were sacrificed by cervical tion 10 days following immunization. ıleens were pooled in a glass tissue · containing 2 ml Hank's balanced salt n (HBSS) adjusted to pH 7.2 with O_3. The cell suspensions were filtered h gauze and adjusted to a concentra- · 6 × 10^7 cells/ml HBSS. To 0.8 ml 0.1 ml spleen cell suspension and 0.1 ne containing 3 × 10^7 SRBC were to yield a final volume of 1 ml con- a 1:5 ratio of spleen cells to sheep cytes. This suspension was vigorously l and immediately placed in a 4° cold for 3 hr. Test tubes containing the s were gently rotated at the end of tion until the sedimented cells were nly resuspended before loading the he- ımeter. Only spleen cells having sur- ntact with a minimum of five red cells ounted as rosettes. Each sample was n triplicate and analyzed in a blind r.

ue-forming cell (PFS) assay. A modi- hnique of the hemolytic plaque assay scribed by Jerne and Nordin (15) was mmunization and LPS treatment were al to that used for the RFC assay. mice in groups of six were used. s were obtained 4 days following im- ıtion and pooled spleen cell suspen- ere adjusted to three concentrations lume: 1:10, 1:100, 1:500) for plating. accurate determination of cell concen- samples of these cell suspensions were and counted by means of a hemocy- r. Two milliliter aliquots of a 1% Noble ılution containing (a) 0.1 ml of 1% dextran, (b) 0.1 ml of a 20% SRBC sion, and (c) 0.1 ml of any of the spleen cell concentrates were evenly on plates containing 10 ml 1.4% so- but prewarmed Noble agar. The ere incubated for 60 min at 37° before guinea pig complement diluted with ronal buffer was added. The plates cubated for an additional 30 min. At ıe the complement was decanted and es were ready to be analyzed. Plates ı of the three concentrations of spleen cells were done in triplicate and analyzed in a blind manner.

Prevention and restoration of radiation-caused immune suppression. The time intervals between LPS injection, 400 r whole-body irradiation, and immunization with SRBC were varied in these experiments.

In the RFC immune response system, the irradiation preceded immunization by 4 days in all experiments. The time of 10 μg ip LPS treatment preceding immunization was varied from −19 days to 0 day. At day 0, the LPS and SRBC were given simultaneously ip, but by two separate injections. The immune response of the animals was measured always 10 days after immunization with SRBC, using the RFC assay.

In the PFC assay, radiation preceded immunization by either 2, 5 or 12 days. The LPS pretreatment was either 2 or 10 days before irradiation. The selection of these two time intervals was a consequence of earlier observations made in our laboratories (16), according to which the optimal time intervals for LPS-induced radiation protection peaked at these two time points. The immune response of the animals was determined by the Jerne plaque assay, always 4 days after immunization.

Whether endotoxic LPS treatment given simultaneously with SRBC immunization can restore damage caused by radiation was determined by giving LPS and SRBC 3 days after total body irradiation with 400 r.

Results. Effect of LPS on the rosette-forming cell immune response to SRBC in normal and irradiated animals. Groups of normal ICR mice (six in each group) were treated with a single dose of 10 μg LPS ip at various time intervals prior to immunization with 1 × 10^8 SRBC. The span of treatment extended from −19 to 0 days. The animals exhibited an enhanced RFC response which was biphasically time-dependent (Fig. 1) and closely paralleled the time-dependent survival of LPS-pretreated, lethally irradiated mice in our previous investigation (16). At shorter time intervals between LPS treatment and immunization, moderate adjuvancy could be seen. If LPS was given on days −7 or −8, suppression became evident. When LPS treatment preceded immunization by 13 days, a period of pronounced adjuvancy was observed.

In order to study the effect of LPS on the RFC response in irradiated mice, a rather high yet sublethal exposure dose of radiation (400 r) was selected. Mice in groups of six were irradiated 5 days before immunization and treated with LPS (10 μg) at identical time intervals as before (Fig. 2). Good enhancement in the number of RFC's relative to irradiated controls was most evident when LPS treatment preceded irradiation by 8–11 days (13–16 days before immunization). A biphasic time-dependent relationship was apparent again. Comparison of the RFC response of the irradiated control group with the non-irradiated controls of the previous experiments showed nearly 80% immunosuppression. However, during the period of maximum LPS enhancement (8–11 days before irradiation), the RFC response for the LPS-treated irradiated mice reached a value of nearly 90% of the nonirradiated controls. Pretreatment of animals with LPS within this time period almost completely prevented the development of radiation-caused immunosuppression.

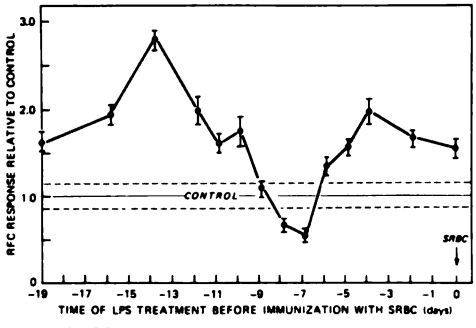

FIG. 1. The RFC response of ICR female mice injected with 10 μg of LPS (*S. marcescens*) at selected time intervals before immunization with 1×10^8 SRBC. Control value for RFC response to SRBC was $2.1 \pm .16 \times 10^4$ RFC per 10^6 spleen cells. Vertical bars represent ±1 SD.

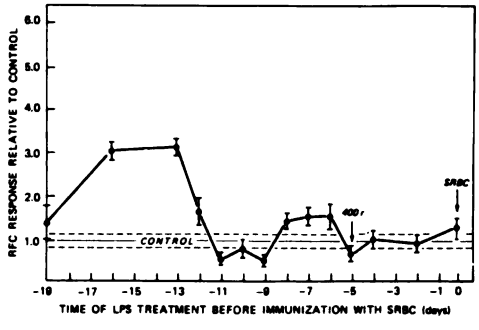

FIG. 2. The RFC response relative to control of ICR female mice injected with 10 μg LPS (*S. marcescens*) at selected time intervals with respect to irradiation (400 r) and immunization with 1×10^8 SRBC. The RFC response to SRBC in irradiated controls (without LPS) was $6.1 \pm .37 \times 10^3$ RFC per 10^6 spleen cells. Vertical bars represent ±1 SD.

Having observed the above described protective effect of LPS when such treatments preceded irradiation, our next aim was to determine if LPS treatment could induce the restoration of the RFC immune response in *previously* irradiated animals. Mice in groups of six were simultaneously immunized and treated with LPS 3 days after receiving 400 r whole-body irradiation (Table I). Comparison was made to one normal and one irradiated control group in order to assess the degree of enhancement of the RFC response in the LPS-treated irradiated group when compared to the irradiated control mice. This represents an immune response which is equivalent to 78% of the nonirradiated control group.

Effect of LPS on the PFC immune response to SRBC in irradiated mice. The results of the experiments in which the effect of radiation on the PFC response was prevented by application of LPS are summarized in Table II. Except for the animals in group 1, all mice showed significant enhancement of the PFC response as compared to animals irradiated and immunized but not LPS treated. The largest enhancement was observed for group 2, in which the LPS treatment preceded irradiation by 2 days and immunization by 7 days. If LPS treatment preceded irradiation by 2 days and immunization by 4 days (group 1 in Table II), the radiation-caused damage could not be prevented.

Discussion. Before beginning our discussion of experimental data it may be worthwhile to justify the use of pooled spleen cell-suspensions within our experimental design. The use of pooled spleen cells has the obvious disadvantage of obliterating the individual variations of animal responses within a given group. In preliminary studies we had determined that the standard deviation of the immune response within most groups was less than 10% of their means. In view of this high degree of consistency we felt justified in pool-

TABLE I. IMMUNE RESTORATION OF IRRADIATED (400 r) MICE BY LPS (*Serratia marcescens*) TREATMENT.

Treatment[a]	Radiation (400 r)	SRBC (1×10^7)	RFC's per 10^6 spleen cells ($\times 10^3$)	Relative to control No. 1	Relative to control No. 2
LPS (10 μg)	+	+	9.7 ± 1.4[b]	$3.2 \pm .88$[c]	$0.78 \pm .20$
Control No. 1 (no LPS)	+	+	3.0 ± 0.7	—	$0.28 \pm .05$
Control No. 2	none	+	12.4 ± 1.2	4.13 ± 1.04	—

[a] Mice were simultaneously immunized and LPS-treated 3 days after irradiation.
[b] Significant at the 0.01 level.
[c] Represents ± 1 SD.

TABLE II. PROTECTIVE EFFECT OF LPS ON THE IMMUNE-RESPONSE OF X-IRRADIATED MICE IN THE PFC RESPONSE.

Group	LPS[a] (on day)	Radiation[b] (on day)	SRBC[c] (on day)	PFC's/10^6 spleen cells[d]	PFC Response Relative to Control[d]
1	0	2	4	2.56 ± 0.19	1.19 ± 0.11
	none	2	4	2.14 ± 0.11	
2	0	2	7	23.11 ± 0.62[e]	5.37 ± 1.11[e]
	none	2	7	4.1 ± 0.88	
3	0	2	14	15.2 ± 1.05[e]	2.38 ± 0.38[e]
	none	2	14	6.5 ± 0.95	
4	0	10	12	7.67 ± 0.57[e]	3.43 ± 0.83[e]
	none	10	12	2.17 ± 0.48	
5	0	10	15	8.65 ± 0.49[e]	4.32 ± 1.12[e]
	none	10	15	2.09 ± 0.53	
6	0	10	22	6.94 ± 0.78[e]	2.36 ± 0.33[e]
	none	10	22	2.93 ± 0.26	
Nonirradiated control				97.4 ± 7.81	

[a] 10 μg LPS ip per mouse.
[b] 400 r whole-body.
[c] 1×10^8 SRBC/mouse.
[d] ±1 SD.
[e] Significant at the 0.05 level.

ing the spleen cells of animals within a given group. The enormous time saving step of pooled cell suspensions provided us with the benefit of an experimental design in which all animals were irradiated on the same day and subsequently sacrificed and assayed on the same day.

Of the many mechanisms whereby endotoxin is supposed to affect the immune system, none offers a satisfactory explanation for the biphasic survival of lethally irradiated animals which we observed in our previous study (16), nor the biphasic RFC response (in normal and irradiated animals), reported here. In the latter assay the remarkable adjuvant effect of LPS given 8–11 days before the immunogen cannot be explained on the basis of any current theories on the mode of adjuvant action.

It is generally accepted that cell proliferation must be an essential feature of the early immune response (17), in which an early radiosensitive phase rapidly moves into a radioresistant phase (18). Endotoxin, by virtue of its antigenic and mitogenic property, may therefore stimulate the antibody-producing precursor cell (B-cell) to differentiate into the less radiosensitive plasma cell (19). It seems likely that LPS affects stem cells, and induces blastogenesis and differentiation, which establish a synchronous cell population undergoing a series of mitotic divisions characterized by cytological, functional and associated radio-resistant changes. That cell synchronization by endotoxin may play a key role in the biphasic protection pattern against lethal doses of radiation is substantiated by the observation that daily repeated LPS treat-

ments provide no radiation protection (20–22).

One cannot exclude the possibility that two different cell types are affected by LPS in both the radiation protection and in the adjuvant effects. One type responds to LPS-induced stimulation relatively quickly and reaches its optimal readiness for irradiation protection or immunization within a few days. The achievement of the same stage of activation takes 8–11 days for the second cell type.

At this time only the above and some additional theoretical possibilities can be offered to explain the multiphasic nature of the LPS-induced enhanced resistance to radiation and enhanced immune response. The great similarities in the two time curves indicate to us eventual interrelationships between the two phenomena, or at least similarities in the chain of events involved in both. Further experiments on the possible mode of action are in progress in our laboratories.

Another point for discussion is the difference in radiosensitivity as observed by the RFC and PFC assays. Although LPS pretreatment conveyed a several-fold increase in both the RFC and PFC response during optimal treatment times, a major difference concerning the radiosensitivities of these two immune responses exists. While a constant exposure dose of 400 r reduced the RFC response of irradiated animals to 30% of the nonirradiated controls, the same dose reduced the PFC response to only 3–7% of the PFC value of the normal, nonirradiated controls. Equally, a disparity exists for the optimally LPS-stimulated groups in their RFC and PFC response when compared to their nonirradiated controls. In contrast to the maximal LPS enhancement of RFC response which attained a value of nearly 90% of the nonirradiated controls, the highest LPS enhanced RFC response in irradiated animals was less than 24% of the nonirradiated controls. The assumption of two separate cell lines for rosetting and antibody production response would conveniently serve to explain the lack of parallelism of LPS radioprotection of the PFC and RFC responses as well as differences in radiosensitivity. It is known that the immunological role of T-cells in expressing cellular immunity is *not* markedly inhibited by irradiation in doses greatly affecting antibody formation (23, 24). That rosette-forming cells are sensitized immunocytes is well established by the simple demonstration that these cells drastically increase in numbers in the spleens of mice immunized with SRBC. However, their precise *in vivo* function as well as their relationship to antibody producing cells remains speculative and controversial. Neonie and Harris (25) postulate that the rosetting cell involves newly synthesized antibody molecules which are still attached to the cell surface and can act as specific receptors for antigen. It has also been shown that: (a) the *in vitro* development of rosette-forming cells requires an active synthetic process; (b) anti-IgG and IgM antibodies inhibit both RFC and PFC assays (26) and (c) RFC's are susceptable to the action of 2-mercaptoethanol to the same extent as IgM antibodies (27). The demonstration of these similarities between specific antibodies and the surface receptors of rosetting cells, however, is by no means conclusive proof that rosetting cells and antibody producing cells are one and the same. It is well known that T-cells also carry antigen-specific surface receptors (28) which are capable of antigen binding (29).

Convincing evidence that PFC's and RFC's are largely independent populations comes from the work of Wilson (30) who was able to separate RFC's from PFC's by means of velocity sedimentation. More convincing were his results obtained by using isolated individual rosette forming and plaque forming cells. As he found, very few RFC's were able to produce plaques. Equally, few PFC's were able to form rosettes. It must also be pointed out that the kinetic profile of the RFC response is entirely different from the PFC response suggesting that RFC's are long-lived immunocytes or memory cells (26).

The final point we wish to emphasize is that it is possible to compensate for radiation-caused immune damage by LPS, but only if LPS is applied at the proper time relative to irradiation. The practical use of such pretreatment in patients undergoing radiotherapy is an obvious aim. How these observations will apply to human cases remains to be explored.

Summary. It has been established that endotoxic lipopolysaccharide (LPS) exerts a

term effect on the immune system. This
: is multi-phasic. Contingent upon the
interval between adjuvant application
immunogen injection, either enhance-
or immunosuppressive effect could be
ved. LPS given 2–4 or 10–16 days before
) red blood cell (SRBC) immunogen
ficantly enhanced the immune response
easured by rosette formation (RFC).
given 7 or 8 days before SRBC was
inosuppressive.
sed on this information, attempts were
: to prevent or repair radiation-caused
inosuppression by using LPS at optimal
It was found that under such conditions
can minimize such damage. The appli-
n of LPS 3 days after irradiation was
arly effective in the repair of immuno-
ression as measured by the RFC assay.
was less efficient but still useful in re-
ig the radiation-caused suppression of
umber of plaque forming cells (PFC).

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Heterogeneity of KCl-solubilized Antigens of Chemically Induced Sarcomas[1] (Immunology) (40052)

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When tested by transplantation techniques chemically-induced tumors appear to carry tumor-unique, noncross reacting transplantation antigens (1–3). In contrast, *in vitro* assays such as microcytotoxicity (4, 5), lymphocyte proliferation (6, 7) and antibody-mediated cytotoxicity (8) show that these tumors also share common antigens in addition to their unique transplantation antigen. Evidence of cross-reactivity between chemically-induced tumors has also been demonstrated through delayed hypersensitivity testing (9, 10) and in certain circumstances by transplantation tests (1, 6, 11).

In previously reported studies from this laboratory (6), solubilized tumor membrane preparations were found to contain antigens which crossreacted among several fibrosarcomas as well as antigens which were unique to each individual tumor. Lymphocyte proliferation assays (LPA) demonstrated these same preparations also contained antigenic activity which, when given with adjuvants, provided tumor specific protection in transplantation tests. Studies presented in this paper show that the biologically active material in such membrane preparations are heterogeneous in molecular size, and that components of different size may either stimulate or inhibit proliferation in lymphoid cell subsets found in the tumor-bearing animal.

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Material and methods. Animals. Female, C57Bl/6J mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. The animals were 6 weeks old upon receipt and were used in experiments when 9–10 weeks old. Animals were fed a diet of Mouse Chow (Purina Co.) and water *ad libitum*.

Tumors. Methylcholanthrene (0.5 mg in 0.1 ml olive oil) was injected into ten animals at four intramuscular sites. Fibrosarcomas become apparent in all mice within 6–12 weeks and were frozen following one passage in syngeneic mice (C57Bl/6J) or were passaged in syngeneic mice for not more than ten passages. Two of these tumors (M3 and M4) were utilized in this study. These tumors were tested extensively and shown to have unique (non cross protective) transplantation antigens, but shared antigens as detected in the LPA (see below). The tumors utilized were not selected on the basis of any known attribute but represented a random selection among those tumors available.

Preparation of solubilized tumor antigens and fractions. A modification of the procedure of Reisfeld (12) as described by Forbes (6) was used to solubilize antigens from sarcomas. The amounts added to cultured lymphocytes were graded by dilution of the crude or fractionated material, and indicated in microliters. Since the character of the activity had not yet been defined chemically, it was not appropriate to express it in terms of protein content. Solubilized tumor membrane preparations were fractionated by ultrafiltration through Amicon filters of varying pore size. Fractions obtained are designated in this report by the molecular weight range contained in the fraction. For example, fraction 50,000–100,000 contained material which passed an XM50 membrane (exclusion limit 50,000 daltons). Fraction <30,000 contained all material which passed a PM30 membrane (exclusion limit 30,000 daltons). That mate-

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ined above a membrane represented which remained after extensive "di- vith large volumes of buffer (phos- affered saline, pH 7.2). The solubi- mor antigen preparations were re- o by a tumor number designation by the bracketed suffix [s]. The membranes used in these procedures XM300, XM100, XM50, and PM30. nes were used according to the man- rs instructions and were only used . The molecular size performance of mbranes is well documented, and d independently in this laboratory ncurrent experiments.

ration of lymphoid cells. Spleen or ode cells were removed from exsan- mice under aseptic conditions. After with scissors in RPMI-1640 (Gibco) were passed through a 60 mesh steel screen followed by sequential through 20 ga, 22 ga, and 25 ga Single cell suspensions were washed ies in RPMI-1640 and were enumer- viable cells by the trypan blue exclu- cedure.

of tumor antigen stimulation of lymph-

The lymphocyte proliferation assay mployed was based upon that de- previously (6), but modified to use ites and automated cell harvesting as d by Hartzman (13). Five hundred l lymphocytes per well in 0.2 ml ontaining 100 μg/ml streptomycin, ml penicillin and 5% heat inactivated serum per ml, were distributed in ottomed Linbro (#IS-MRC-96) mi- . The antigens or dilutions thereof led in a volume of 20 μl. The cultures ubated at 37°C in an atmosphere of . Tritiated thymidine (0.5 μCi) was o each well after 48 hr incubation. ig 18 hr additional incubation the were harvested on glass fiber filters harvester. The samples were then d and counted in a Beckman LS250 intillation counter. Data presented ted the mean cpm values plus or ne standard error (SE) of four repli- ures at each dose tested.

h experiment lymphocytes of tumor- animals were taken from the animals following an injection of 1×10^5 viable tumor cells intramuscularly. Tumor cells for injection were prepared as previously described (14). Normal lymphocytes were taken from normal, age matched mice.

Results. Chemical characterization of lymphocyte stimulating activity in KCl solubilized tumor membranes requires initial purification of large volumes of material. Ultrafiltration through selected membranes provided a means to separate large volumes into fractions which were suitable for further characterization. In addition, these fractions were found to have selective effects on lymphocyte subpopulations. Unfractionated 3 *M* KCl solubilized tumor preparations of murine fibrosarcomas are often viscous, difficult to filter to assure sterility, and inactive in a lymphocyte proliferation assay, possibly due to content of polymeric DNA. It was found initially that a single purification step passage through ultrafilters excluding material over 300,000 daltons usually yielded a nonviscous fraction which contained the material stimulatory in the LPA. However, some lots of antigen were inactive after such a preliminary step (see Fig. 2A).

This note describes an extension of this approach in which fractions which were or were not inactive after this initial step were further fractionated and then tested for a capacity to stimulate spleen of tumor-bearing animals. Data from a representative experiment are shown in Figs. 1 and 2. Spleen cells were stimulated in a double peak pattern by unfractionated solubilized material (Fig. 1A). A major, broad peak occurs at the high (10 μl) dose and a second peak is found in the low (1 μl) dose range. The second peak was characterized both by a narrow range of effective dose and smaller magnitude, as compared with the high dose peak. Fractions containing material under 100,000, 50,000, or 30,000 daltons, respectively, gave reduced capacity to stimulate spleen cells especially that below 30,000 (Fig. 1D). In some experiments, the low dose peak persisted in this latter fraction (see Fig. 3, for example) suggesting that the active fraction has a lower limit of molecular size on the order of 30,000.

Preparations inactive after passage through the XM300 membrane were further fractionated by extensive dialysis on XM100 and XM50 membranes, which yield preparations

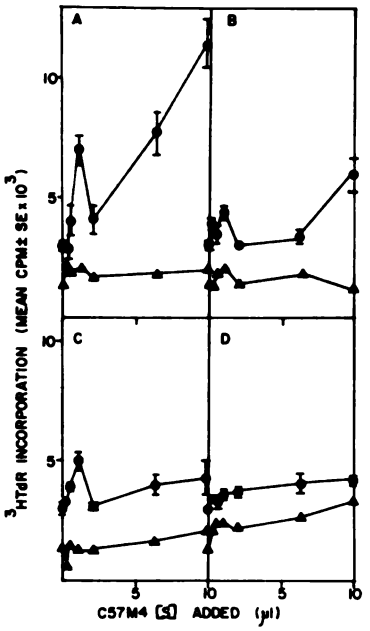

FIG. 1. LPA data from culture of spleen cells taken from normal (▲) or M4-bearing (●) animals, to which varying amounts of: (a) Unfractionated M4-[s]. (b) M4-[s], <100,000. (c) M4-[s], <50,000. (d) M4-[s], <30,000, had been added. Data represents one means of four replicates ± SE.

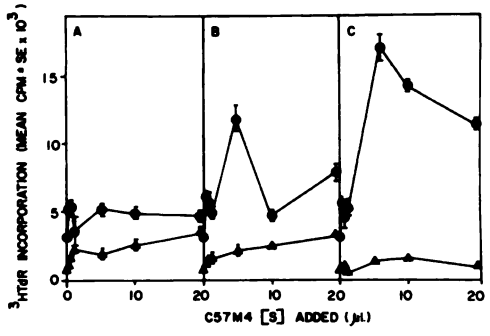

FIG. 2. LPA data from spleen cells taken from normal (▲) of M4-bearing (●) animals to which had been added varying amounts of (a) M4-[s], <300,000. (b) M4-[s], 100,000–300,000. (c) M4-[s], 50,000–100,000.

having both upper and lower limits of molelcular size. Activity in such preparations were greatest in the 50,000–100,000 dalton fractions. A representative experiment is shown in Fig. 2.

Unfractionated solubilized tumor membranes derived from syngeneic tumors contained lymphocyte stimulating activities shared by some but not all syngeneic MCA

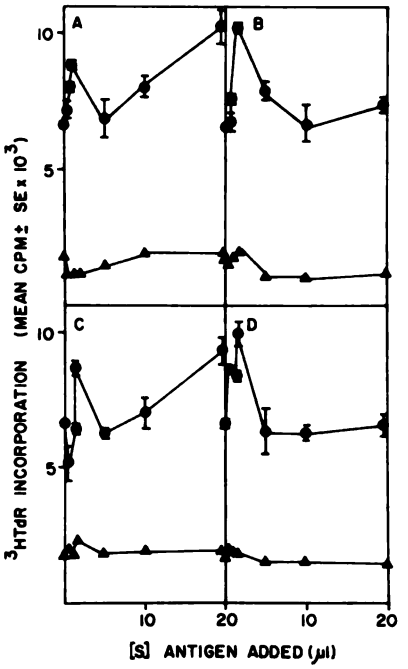

FIG. 3. LPA data from spleen cells taken from normal (▲) or M4-bearing animals (●), to which had been added varying amounts of (a) M4-[s], 30,000–300,000. (b) M4-[s], <30,000. (c) M3-[s], 30,000–300,000. (d) M4-[s], <30,000.

tumors (6, 7). It seemed possible that such crossreacting antigens might segregate into fractions of differing molecular size, and thus permit identification of a tumor-unique antigenic moiety. Figure 3 represents data from experiments in which fractions from two tumors were tested simultaneously. That containing the higher (30,000–300,000 daltons) or lower (<30,000 daltons) molecular weight molecules derived from the M3 or M4 tumor gave virtually identical patterns of stimulation of spleen cells taken from animals bearing the M4-tumor. Thus, no differences in the molecular size spectrum difference of the antigenic material was detected by this approach.

Discussion. The data presented here define further some characteristics of the lymphocyte stimulating material present in 3 *M* KCl-solubilized methylcholanthrene-induced fibrosarcomas. Application of a graded filter technique showed that stimulatory moieties involved are of two general categories in molecular size. One is about 30,000 daltons and

her 50,000–100,000 daltons. The latter
ctive at a high dose level; the crude 3M
olubilized membrane preparations also
r to contain both high (>300,000) and
<50,000) molecular weight inhibitory
nces. Whether these instances were
ic or nonspecific was not determined.
oncomitant presence of both antigens
stimulate lymphoid cells and inhibitors
nown character obviously complicates
terpretation of any experimental data
ed with unfractionated material.
ntitative aspects of the dose response
s, the two peak stimulatory behavior,
ave at least two explanations. For ex-
, two specific lymphocyte subpopula-
nay be responding to the high and low
ular weight fractions. On this premise,
olecular weight stimulation gives a
peak, with inhibition at higher dose
reminiscent of *in vitro* T-cell responses
ogens and antigens (15, 16). The high
timulation peaks are sustained over a
er range of dose, or show progressively
magnitude. The latter patterns suggest
ative activation of multiple cell subsets,
specific polyclonal activation of B-cell
s by B-cell mitogen (17). It is estab-
that both T- and B-cell subsets are
ated by solubilized tumor preparations
lymphocyte proliferation assay (unpub-
), and that a third, non-T, non-B cell,
acrophage subset ("null cells") is also
ated by such antigens. This explanation
viable, but not established by any data
herein.
econd explanation might be that the
ose stimulatory material is present in
preparations in a concentration signif-
higher than that giving high dose
ation. Possibly responsible for the latter
be that the material is in polymeric
exes.
presence of inhibitors of uncertain
city is an observation of considerable
al importance in refining the LPA into
e useful experimental and clinical (18)
hese data include that a relatively sim-
rification step can yield stimulatory
tumor antigen or more uniform quality.

Summary. Solubilized antigens of murine methylcholanthrene-induced fibrosarcomas prepared by treatment of tumor tissue with hypertonic KCl were separated into fractions depending upon exclusion by molecular weight by selected ultra-filters. These studies showed that material of both high molecular weight (>300,000) and low molecular weight (<30,000) inhibited ^{3}H-thymidine incorporation by lymphoid cells taken from tumor-bearing animals. When the inhibitor were removed, the material remaining stimulated proliferation in such lymphoid cells over a wide range of molecular weights (<30,000 daltons to >100,000 daltons).

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Increased ATPase and Decreased Alkaline Phosphatase Activities by Parathyroid Hormone in Cultured Chick Embryo Tibiae[1] (40053)

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Bone alkaline phosphatase (AP) activity has been shown to be highest in areas of bone undergoing mineralization (1, 2), but its role and substrate(s) are not known. Magnusson *et al.* (6) performed histochemical studies which suggest that bone AP in mineralizing areas might be an alkaline ATPase that uses Ca-ATP as substrate. In contrast to this concept, Messer *et al.* (7) have shown evidence that bone nonspecific AP and ATPase might be properties of different enzymes.

The effect of parathyroid hormone (PTH) on bone AP activity has previously been studied *in vitro* with conflicting results. Compared to bones cultured without PTH, mouse calvaria incubated with the hormone have been shown to contain increased AP activity (8), decreased AP activity (9), and unchanged AP activity (10). Isolated rat bone cells incubated with PTH in the medium showed a 40% decrease in surface AP activity after only 2 hr (11). Luben *et al.* (12) have isolated a population of osteoblastlike cells from mouse calvaria. Incubation of these cells with PTH causes a substantial decrease in AP activity after 24 hr. Using embryonic chick tibiae in organ culture (13, 14), the present study was undertaken to determine and compare the effects of PTH on bone AP activity and on bone ATPase activity. Different effects of PTH on the two activities would be consistent with the concept of two separate enzymes.

Materials and methods. Organ culture. Tibiae were aseptically removed from 10- to 12-day old White Rock chick embryos and rinsed briefly in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid)-buffered salt solution, pH 7.4. The tibiae were then placed in 25 ml Erlenmeyer flasks containing 3–5 ml chemically defined culture medium. The flasks were gassed with 5% CO_2 in air, closed with silicone rubber stoppers, and placed on a rocker platform in a 38° incubator. All culture media were prepared by modification of Eagle's minimum essential medium as described by Ramp and Neuman (13). The calcium and phosphate concentrations were 1.8 m*M* and 1 m*M*, respectively. Bovine serum albumin (1 mg/ml) was added to the media to prevent the PTH from adsorbing to the glass culture flasks. Tibiae were paired between control and treatment flasks such that each embryo served as its own control. The bones were precultured for 24 hr in medium without PTH to remove extraneous AP (enzyme in blood or in the small amounts of non-bone tissue explanted with the bone). Preculture removed approximately 25% of the AP associated with noncultured bones. All of the AP activity lost from the tibiae during preculture was recovered in the medium. After preculture the medium was changed to fresh medium containing either PTH or the PTH vehicle. The incubation was then continued for 24 or 48 hr. At the end of the culture period, the bones were briefly rinsed in 0.9% NaCl solution. Bones and media were frozen and stored at −20° until use.

Unless otherwise noted, the source of PTH was Lilly Parathyroid Injection. Highly purified bovine PTH was obtained from Inolex Laboratories and the synthetic 1–34 peptide of bovine PTH from Beckman. The vehicle for Lilly Parathyroid Injection was 0.2% phenol, 1.6% glycerin, and the vehicle for the purified and synthetic PTH was 0.001 *N* HCl, 0.9% NaCl.

Analytical procedure. Bones were homogenized in distilled water with a Polytron homogenizer (Brinkmann). The homogenate and culture media were assayed for AP activ-

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ity at pH 9.8 using *p*-nitrophenylphosphate (*p*-NPP) as substrate (15). One unit of enzyme activity is defined as one micromole of substrate hydrolyzed per hr at 38°, and enzyme activity is expressed in terms of activity units (A) per bone. Bone ATPase activity was determined using the supernatant of the bone homogenate after centrifugation at 1000*g* for 10 min to remove large particles. Samples of supernatant were incubated for 1 hr with 3 m*M* ATP containing γ^{32}P-ATP (SA = 0.2 Ci/mole), 3 m*M* $MgCl_2$ in 75 m*M* Tris, pH 8.5, in a total vol of 0.2 ml. In some assays 5 m*M* levamisole (American Cyanamid Co., Princeton, NJ) was included in the hydrolysis incubation. The rate of hydrolysis of ATP in this assay was maximal at pH 8.5, proceeded at a constant rate during this time period, and was proportional to the amount of homogenate assayed. The hydrolysis was stopped with 0.2 ml 10% trichloroacetic acid. Free P_i was extracted with 0.1 ml 5% ammonium molybdate and 0.4 ml isobutanol. After mixing for 30 sec, the samples were centrifuged at 2000*g* for 10 min to separate the aqueous phase from the isobutanol (16). The free P_i in a sample of the isobutanol phase was calculated from the amount of ^{32}P as determined by liquid scintillation counting and ATPase activity is expressed as nmole of ATP hydrolyzed per bone per hr. Lactate released to the medium was measured enzymatically (17) after removal of interfering substances with Norit A charcoal. The significance levels of differences between paired control and treated bones were determined using Student's *t* test for paired data. A value of $P < 0.05$ was considered significant. Since this test of significance is based on the difference between paired bones, no inference as to the levels of significance can be made from the standard error of mean values. Therefore, these were not included in the figures.

Results. The experiment shown in Fig. 1 was performed to determine the changes in total AP activity (bone content + medium content) of tibiae during 48 hr of organ culture. Tibiae from 11-day embryos were precultured for 24 hr. One bone from each pair was then frozen, and the other was placed in fresh medium and incubated for 48 additional hr. During the 48-hr incubation, the bone content of AP activity increased 35% compared to the preculture level. When the released activity was added to the bone content, the total amount of AP activity after 48 hr of culture was 171% of the preculture activity. The most likely explanation for these data is that the tibiae were actively synthesizing AP *in vitro*. However, the possibility of activation of already synthesized enzyme cannot be ruled out.

The data in Fig. 2 show that while tibiae

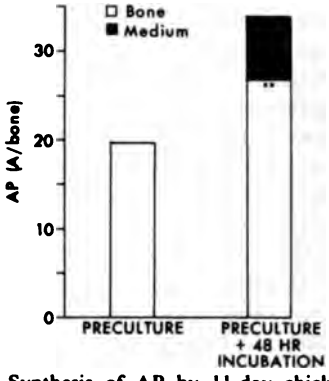

FIG. 1. Synthesis of AP by 11-day chick embryo tibiae. Paired bones were precultured for 24 hr. One bone from each pair was then frozen and the other was cultured an additional 48 hr in fresh medium. Bone homogenates and the 48 hr incubation media were assayed for AP. Each bar represents the mean value for eight tibiae. $^{**}P < 0.01$ compared to paired precultured bones.

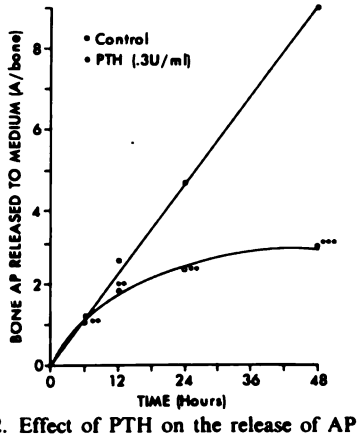

FIG. 2. Effect of PTH on the release of AP by 12-day chick embryo tibiae in organ culture. Bones were paired between media containing control and PTH (0.3 U/ml) media. Serial samples of media were collected during the 48 hr incubation (after preculture) to monitor the release of AP. Each point represents the mean value for five tibiae. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to paired control bones.

cultured in control medium released AP at a constant rate, PTH (0.3 U/ml) caused a decrease in released activity. After 6 hr the AP activity in the medium of PTH-treated bones was significantly lower than in control medium according to the paired *t* test, even though the difference between the means is quite small. By the end of the 48-hr culture period the PTH medium contained only 36% of the control activity. Addition of PTH to medium containing AP did not alter the AP activity, indicating that there was no direct inhibition of the AP enzyme by PTH (data not shown).

The effect of PTH to decrease the amount of AP activity released by tibiae was reflected in the bone content of AP activity. Figure 3A shows the bone content of AP after 48 hr of culture in medium containing three doses of PTH: 0.003, 0.03, and 0.3 U/ml. The decrease in bone content (Fig. 3A) and release (Fig. 3B) of AP caused by PTH is related to the concentration of PTH in the medium, with a concentration as low as 0.03 U/ml resulting in more than a 30% decrease in AP activity after 48 hr.

Three different preparations of bovine PTH (parathyroid extract, highly purified hormone, and the 1–34 peptide fragment) all caused similar decreases in AP activity. Incubation in medium containing 1 U/ml of either highly purified or synthetic hor caused the same 60–70% reduction of bone content and release of AP activ observed with parathyroid extract.

Studies demonstrating that bone Al hydrolyze ATP (18, 19) have led to the posal that bone AP may function as an ase, possibly to provide energy to increa efflux from bone cells (5). Because i clear that PTH decreased the AP activ embryonic chick tibiae (Figs. 1–3), the e iment shown in Fig. 4 was perform determine whether the bone ATPase ac was similarly decreased. As in previou periments, bones were precultured in co medium for 24 hr and then cultured for in medium containing either PTH (1 U or vehicle. The ATPase assay was perfo both in the presence and absence of le sole (5 m*M*), an inhibitor of nonspecific AP (20, 21). At this concentration, levan caused total inhibition of the bone no cific AP assayed using *p*-NPP (data shown). At the end of the 48-hr cultur riod, the total ATPase activity of PTH-tr bones was the same as that of the co bones (Fig. 4B), in contrast to the AP ac of PTH-treated bones which was again r edly decreased (Fig. 4A). When the AT activity due to nonspecific AP was elimi by including levamisole in the ATPase

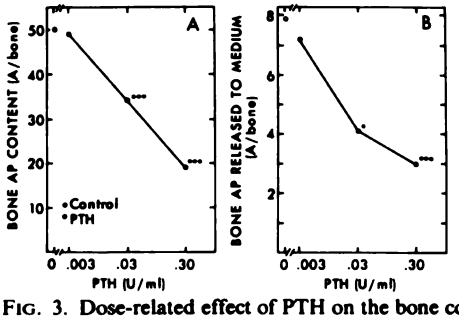

FIG. 3. Dose-related effect of PTH on the bone content (A) and release (B) of AP. Tibiae from 12-day chick embryos were paired between control medium (open circles) and PTH-containing medium (closed circles). After preculture and incubation for 48 hr, bone homogenates and culture media were assayed for AP activity. There was no significant difference in either the bone content or release of AP by control bones in different dose groups. Thus, these values were pooled and are represented by a single open circle. Each closed circle represents the mean value for five tibiae. * $P < 0.05$, *** $P < 0.001$ compared to paired control bones.

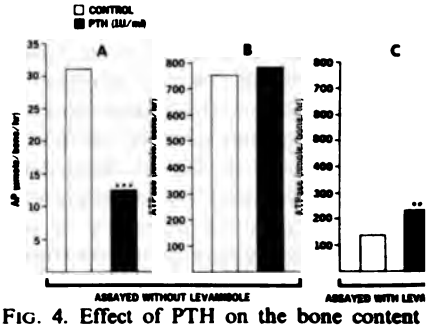

FIG. 4. Effect of PTH on the bone content (A), total ATPase (B) and ATPase in the presen mM levamisole (C). Tibiae from 11-day embryc incubated in medium containing PTH (1 U/ml) hr after a 24 hr preculture. Enzyme activity is ex as quantity of substrate hydrolyzed per bone pe that comparisons can be made between AP and A activity. The mean (±SE) of the differences betw ATPase activities of paired, PTH-treated and bones (C) is 87 ± 23. Each bar represents the mea of 8 tibiae. ** $P < 0.01$, *** $P < 0.001$ compared to control bones.

the ATPase activity of PTH-treated bones was 160% of the activity of control bones (Fig. 4C). Three additional experiments similar to the one shown in Fig. 4 have confirmed this finding. The data shown in Fig. 4 indicate that bone contains at least two phosphatase activities at alkaline pH: a nonspecific alkaline phosphatase (AP) that is decreased by PTH, and an ATPase that is increased by PTH.

Discussion. This study demonstrates for the first time, by using a selective inhibitor, the existence of an ATPase activity in bone which can be distinguished from nonspecific alkaline phosphatase. In contrast to AP activity, which was decreased in tibiae cultured in medium containing PTH, this specific ATPase was shown to be substantially increased in PTH-treated bones.

Bone AP, the enzyme inhibited by levamisole, has been associated with mineral deposition since 1923 when Robison discovered an enzyme in ossifying cartilage which would hydrolyze hexosemonophosphoric acid esters, and suggested a role for this enzyme in bone calcium phosphate deposition (1). Since that time, alkaline phosphatase has been localized in osteoblast plasma membranes and matrix vesicles in areas undergoing mineralization (2). In spite of the fact that AP occurs in mineralizing areas and seems to be necessary for the process of mineral deposition, no mechanism for this process has been clearly demonstrated. Nevertheless, the inhibition of bone AP activity by PTH suggests that PTH may exert an acute inhibitory effect on bone mineral deposition.

The present study did not determine whether the decrease in AP activity caused by PTH was due to inhibition of synthesis of new enzyme, or to the inhibition of the activity of the enzyme. Work with HeLa cell AP has suggested that cellular cAMP may be involved in regulating AP activity (22). In HeLa cells, agents leading to an increased intracellular cAMP concentration also caused decreased AP activity. It is possible that the bone cell cAMP which is increased by PTH (23) may be responsible for the decrease in AP activity.

Studies of alkaline phosphatase from rat odontoblasts involved in dentin formation have demonstrated the presence of a nonspecific AP, as well as a Ca-ATPase (21), which is active in the presence of 0.5 m*M* levamisole. Rat odontoblast AP activity was nearly completely inhibited by 0.5 m*M* levamisole, while the chick bone AP in the present study was only partially inhibited by levamisole concentrations less than 5 m*M*. The difference between the concentrations of levamisole required to inhibit mammalian bone AP and avian bone AP is consistent with a recent report by Reynolds and Dew (24) which showed that levamisole was ten times more potent as an inhibitor of mammalian bone AP than as an inhibitor of avian bone AP. As in the present study, they showed that 5 m*M* levamisole resulted in near total inhibition of avian bone AP.

It has been suggested (21) that in odontoblasts, the AP functions to remove mineralization inhibitors such as inorganic pyrophosphate and to increase the local phosphate concentration while the Ca-ATPase acts as a Ca^{2+} transporting enzyme to accumulate Ca^{2+} ions in the cells. These two odontoblast enzymes may be analogous to the two phosphatases studied in the present work. However, the increase in bone ATPase that resulted from culture from PTH suggests that the ATPase may function in PTH-stimulated Ca^{2+} efflux from bone cells rather than in accumulation of Ca^{2+} for mineral deposition. Ramp (5) has shown that an early effect of PTH is to increase the efflux of calcium from bone to medium without increasing the P_i release. It was suggested that this apparent stimulation of "Ca pumping" might be due to an increased ATPase activity. The ATPase that is not inhibited by levamisole and is increased in PTH-treated bones may be this enzyme.

Summary. Addition of PTH to the medium of embryonic chick tibiae in organ culture caused a decrease in AP activity. Total ATPase activity was the same in control and treated bones, but ATPase activity assayed in the presence of levamisole, an inhibitor of bone AP activity, was greater in PTH-treated bones. These data suggest that there are at least two different phosphatase activities in bone with optimal activity at alkaline pH: a nonspecific alkaline phosphatase which is decreased in PTH-treated bones, and an ATPase which is increased by the hormone.

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Effects of Acute Fluoride Intoxication on Rats[1] (40054)

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overall effects of acute fluoride poison- undoubtedly related to the concentra- fluoride in circulating plasma and in ;ues. The minimum concentration of e in plasma that will result in death is ly dependent on a number of factors s the mode of intake, duration of ex- elevation in fluoride concentration, age animal, the critical level that affects es, and the relation of the fluoride tration to that of other ionic constitu- the plasma and tissues.

lethal concentration of fluorine in of fasted rabbits (1), acutely poisoned ric intubation of a solution of sodium e, (100–140 mg/kg body weight) was (≤28 ppm) than that reported by De *et al.* (2) for young rats given 50 mg/kg y weight (10–12 ppm). The highest fluoride concentration (35 ppm) ob- by Hall *et al.* (1) was found 2 hr after t was fatally poisoned with 140 mg of fluoride per kg of body weight. Fu- and Okuda (3) found levels of 20 ppm e in the plasma of rabbits one hr after I administration of a single dose of 220 ium fluoride and at the end of the 24- od the concentration had returned to levels. A decrease in total calcium tration in plasma was observed while e levels increased following the appli- of 25 mg sodium fluoride per kg of veight to the buccal mucosa of young

rder to establish the range of plasma e concentrations that occur as a con- ce of acute fluoride poisoning, young its were employed in this investigation y the uptake and distribution of par- ly administered high fluoride doses. fects of these treatments on the ionic and total calcium concentrations in plasma were also examined since acute fluoride poisoning may produce alterations in plasma calcium levels and interference with the role of calcium in metabolism.

Methods. Male rats weighing approximately 200 g were given 3, 4 or 5 mg of fluoride (15, 20 or 25 mg/kg body weight) as sodium fluoride intraperitoneally in 1 ml of solution. The rats were fed Purina Laboratory Chow (20–60 ppm F^-) and tap water (1 ppm F^-) throughout the experimental period. A few select animals were given inert fluoride labeled with radioactive fluoride (^{18}F), a positron emitter with a half-life of 109.7 min and sacrificed 10, 30 or 60 min following the dosage. All animals receiving 3 or 4 mg of fluoride were bled under ether anesthesia by heart puncture at 10 min and 1, 4 and 24 hr after the dosage. Select animals receiving radioactive fluoride were also bled after 30 min. To test the possibility that food residues present in the intestinal tract and stomach of animals given access to food might function as a significant compartment for distribution of intraperitoneally administered fluoride or modify the toxic effect of acute doses of fluoride, some of the animals were removed from food and water 16 to 18 hr prior to being given the fluoride challenge dose labeled with radiofluoride (^{18}F). The animals given 5 mg of fluoride immediately became moribund and blood could be obtained only at 10 and 60 min after the dosage since most animals did not survive for longer periods. Anesthesia was generally not required for animals receiving 5 mg of fluoride. In addition to plasma, the humeri, tail tendon and muscle were taken for analytical and for radiochemical analyses from animals receiving labeled fluoride. Control animals that did not receive the fluoride treatment were sacrificed to provide baseline information on the normal concentration of fluoride in plasma and the selected tissues.

The total fluorine concentration of blood

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plasma from each animal was determined by microdiffusion and colorimetric analyses (4). Ionic fluoride levels were measured in plasma ultrafiltrates with the Orion ion-specific electrode (5) when sufficient volume of plasma was available. The total calcium levels in plasma were determined using a modified calcium fluorimetric technique (6). The ionic calcium concentrations were determined with an Orion flow-thru calcium ion-activity electrode. Radiofluoride concentrations in the plasma and other tissues were determined with a well-type crystal scintillation counter. All radioactivity measurements, after correction for background counts and volume, were adjusted for radioactive decay to a single point in time.

Results. Rats receiving 3 or 4 mg of fluoride (15 or 20 mg/kg body weight) exhibited extremely high mean plasma fluoride levels after 10 min which declined rapidly thereafter (Table I). By 24 hr plasma fluorine concentrations had returned to nearly preexperimental levels (0.12 ± 0.009, 12 animals). After 10 min those animals given the 5 mg dose exhibited plasma fluorine levels as high as 57 ppm. The concentration of fluorine found in plasma, tendon and humeri of a limited number of the animals that received the dose labeled with radiofluoride and were sacrificed at 10, 30 and 60 min following the challenge are displayed in Table II. These tissues and the muscle were analyzed for radiofluoride. Calculated fluoride levels were obtained based on the ^{18}F determination of the tissue and the specific activity (cts/μg ^{19} administered solution. The fluoride tration in the muscle of these animal relation between the muscle and pl oride contents are presented sepa Table III.

The concentrations of ionic and cium in the plasma are presented in bar diagrams. The number at the to bar indicates the number of anima mean value. The total height of the cates the total plasma calcium conc (mg %) and the horizontal line near t of the bar denotes the mean concent ionic calcium (mg %). The numb clear area of the bar is the % of calcium in the ionic state.

Discussion. Animals receiving 3 o fluoride (15 or 20 mg/kg body we spite having very high plasma fluor (>30 ppm) 10 min after receiving lenge dose, invariably lived and ex rapid return toward the preexp plasma total fluorine level (0.12 p proximately 70% of the plasma tota was ionic.

The animals given 5 mg of fluorid always have higher plasma ionic flu els than those receiving the 4 mg d is particularly evident in the results There is no simple explanation wh the animals given the 5 mg dose di 60 min whereas all the animals tre 4 mg of fluoride survived. It is indee that much higher levels than 49 an

TABLE I. FLUORIDE CONTENT OF PLASMA AFTER INTRAPERITONEAL ADMINISTRATION OF FLU

Time of collection	Fluoride dose[b] 3	4	5
10 minutes			
Total	29.5 ± 1.58 (12)	35.1 ± 2.52 (12)	48.0 ± 2.1
Ionic	22.9 ± 1.07 (9)	29.2 ± 2.45 (9)	37.6 ± 2.8
60 min			
Total	6.4 ± 0.75 (11)	15.1 ± 1.08 (14)	9.0 ± 2.4
Ionic	5.2 ± 0.90 (11)	11.9 ± 0.079 (8)	4.17 ± 1.8
4 hr			
Total	0.72 ± 0.108 (10)	1.41 ± 0.253 (10)	
Ionic	0.43 ± 0.020 (4)	1.44 ± 0.112 (4)	
24 hr			
Total	0.19 ± 0.031[c] (11)	0.33 ± 0.105 (8)	
Ionic	0.05 ± 0.006 (3)	—	

[a] Parts per million ± SEM (number animals).
[b] mg/200 g body weight.
[c] Preexperimental levels 0.12 ± 0.009 (12).

TABLE II. FLUORIDE CONTENT OF TISSUES[a] OF RATS AFTER ADMINISTRATION OF LARGE DOSES OF FLUORIDE LABELED WITH RADIOFLUORIDE.[b]

Fluoride[c] injected	Plasma		Tendon		Humeri	
	Predicted	Found	Predicted	Found	Predicted	Found
			10 min			
3	25.0	24.7	12.0	10.0	191	160
	21.1	21.8	11.1	9.6	181	172
4	37.7	34.3	13.9	11.4	203	174
	22.9	22.6	34.3	30.4	163	174
5	46.6	43.2	15.5	13.4	214	189
	54.6	49.1	13.5	12.3	227	228
			30 min			
3	15.1	14.1	19.5	20.0	245	232
	13.6	13.0	8.1	6.7	242	232
4	23.7	20.3	7.5	5.1	271	253
	21.3	20.7	13.1	11.3	263	301
5	27.6	27.6	18.5	21.6	288	243
	27.1	21.7	16.0	14.4	283	287
			60 min			
3	7.3	6.4	4.8	4.4	248	257
	2.5	2.6	2.8	3.0	197	188
4	14.1	12.5	8.7	7.6	305	271
	11.7	10.2	9.1	7.3	334	270
5	13.6	12.8	10.5	9.1	279	346
	17.1	17.8	12.5	12.6	269	316

[a] Parts per million on fresh weight or volume basis.
[b] The predicted values for fluoride contents were derived from the ^{18}F contents of the plasma and tissue, the total ^{18}F and ^{19}F body contents and the dose of fluoride given the animals.
[c] Milligram fluoride ion as sodium fluoride.

TABLE III. FLUORIDE CONTENT OF MUSCLE.[a]

Time (min)	Dosage[b]					
	3		4		5	
	ppm	% Plasma[c] level	ppm	% Plasma level	ppm	% Plasma level
10						
1	5.1	20.4	6.8	18.0	9.0	19.3
2	4.8	20.7	5.2	22.7	9.3	17.4
30						
1	4.2	27.8	6.8	28.7	10.0	36.2
2	4.2	30.9	7.6	35.7	10.0	36.9
60						
1	2.7	37.0	5.2	36.9	8.5	62.5
2	0.7	28.0	5.2	44.4	9.5	55.6

[a] Based on specific activity of ^{19}F solution injected and the ^{18}F content of muscle.
[b] Milligram/200 g body weight.
[c] Determined level.

for total and ionic fluorine concentration, respectively, observed for individual animals may have existed prior to or shortly after the 10 min sampling period following the 5 mg dose of fluoride. A large excursion in fluoride content of body fluids could produce alterations in metabolism for which these animals were not able to fully compensate and, as a result, death occurred within 60 min. The *in vitro* activity of a number of enzymes has been reported to be reduced to one-half or less by concentrations of fluoride between 0.2 and 19 ppm in the media (7). These concentrations are considerably higher than the ionic fluoride concentration found in plasma and soft tissues under normal circumstances (0.01–0.05 ppm) but in some instances are not higher than the levels attained after the large doses of fluoride. De Lopez *et al.* (2) used young animals that were fasted for 24

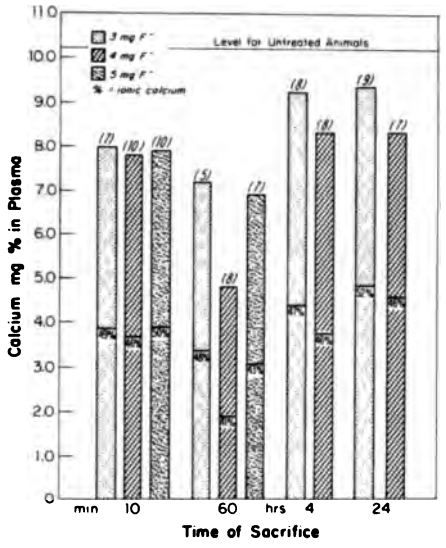

FIG. 1. Effect of acute fluoride toxicity on plasma calcium levels. The number at the top of each bar indicates the number of animals in the mean value. The total height of the bar indicates the total plasma calcium concentration (mg %) and the horizontal line near the center of the bar denotes the mean concentration of ionic calcium (mg %). The number in the clear area of each bar is the % of the total calcium in the ionic state.

hr before being given, by intubation, higher fluoride doses (40–70 mg/kg) than those employed in this study (15–25 mg/kg). The method of administering fluoride will influence the level of fluoride attained in the plasma. Interperitoneal administration of fluoride results in a rate of entrance of fluoride into the plasma which is only slightly slower than that obtained with intravenous administration. Fluoride given by intubation is evidently absorbed more slowly since lower peak plasma concentrations (10 ppm) were observed in De Lopez's study than in the present study. De Lopez *et al.*, however, reported plasma fluoride levels of approximately 4 ppm 12 hr after the administration of the dose, whereas in this study, levels of less than 2 ppm were observed within 4 hr. It is possible that a plasma fluoride level of 4–10 ppm for a 12-hr period is more toxic than higher levels for a considerably shorter period of time. There is little, if anything, known as to the *in vivo* effects of large increments in fluoride ion concentration on enzymatic activity. Numerous changes in the concentrations of glycolytic and citric acid cycle intermediates in the liver of intact rats given acute doses of fluoride have been reported. There was no indication of enzyme inhibition in the kidney (8). With the exception of an increase in citrate levels in soft tissues, the changes were shown to be secondary to a primary effect of dietary fluoride on the amount and pattern of food intake (9). Recently, Ferguson reported that 10 ppm of fluoride provided in the drinking water for 12 weeks resulted in a decrease in serum alkaline phosphatase activity in rats, but no change in the activity of this enzyme in the liver or intestinal tissue (10). Acid phosphatase, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase and leucine aminopeptidase activities in serum, liver, kidney or intestine were not influenced by this level of fluoride intake. In a similar study with rabbits little change, if any, in enzyme activities was noted (11).

To test the possibility that food residues in the intestinal tract and stomach might act as a significant compartment for distribution of the fluoride administered intraperitoneally, a group of animals not fed for 16–18 hr prior to experimentation were given the fluoride doses labeled with radiofluoride. The quantity of administered radiofluoride found in the combined contents of the stomach and intestinal tract of fed animals, which can be considered to contain a pool of inert fluoride, was small (less than 2.5% of the dose). It was therefore, concluded that the contents of the gastrointestinal tract was not an important factor acting to sequester fluoride administered intraperitoneally.

Within 10 min after the animals were given fluoride the total calcium level in the plasma was depressed in all dosage groups, although the % of the total calcium in the ionic form remained normal (Fig. 1). At 60 min a further reduction in total calcium in plasma was observed with all animals, but by four hr the trend was reversed for the animals dosed with 3 and 4 mg (15 and 20 mg/kg body weight) and calcium levels had risen considerably and plateaued at a level slightly below the normal concentration for untreated animals (10 mg %). The animals receiving 5 mg of fluoride did not survive for longer periods of time. The ionic calcium levels were severely depressed by 60 min. These results for the first 60 min are similar to the observations of Fujimoto and Okuda (3) who found that rabbits given a large oral dose of fluoride

ıg/kg body weight) had a slight de- in plasma calcium levels after 3 hr was still apparent 21 hr later. In their animals receiving 25 mg of fluoride exhibited an immediate sharp decrease ma calcium concentration of approxi- 25% by one hr which was followed by progressive decrease for the remainder 24-hr period to reach a level 30% lower ormal. In the present study, however, mals receiving 3 or 4 mg (15–20 mg/kg veight) showed good recovery by 4 hr. total and ionic plasma calcium con- ions found 30 and 60 min following se would not seem to be compatible fe and normal neuromuscular activity basis of accepted concepts. Neverthe- l the animals given 3 or 4 mg fluoride d these circumstances. Surprisingly, s receiving 5 mg of fluoride exhibited total and ionic calcium levels in their at one hr than the animals given 4 mg ıride. A possible explanation for the at animals receiving 4 mg of fluoride ble to survive and rebound from their y and altered calcium status is that eneral metabolism was so severely af- but not irreversibly, that their life ıes were operating at a minimal rate. such circumstances, energy production ıscle contraction would be greatly re- and obvious tetany would not be ob- Tetany was not observed in this study. ıtrast, the animals that received 5 mg ride had proceeded past the point of bility and were so severely poisoned e soon ceased. The inhibition of critical e system(s) may have been responsible th, but this, like the explanation above, a speculation.

e was good agreement in the predicted lculated) and determined total fluoride in all tissues taken from the animals adiofluoride with the possible excep- the concentration found in the humeri ın (Table II). Undoubtedly, the agree- etween the results for fluoride contents d by the two procedures is influenced fact that the fluoride contents were uch elevated over the normal concen- and any bound or nonexchangeable e was a relatively small part of the ıorine content of the tissues. To some this concordance of results lends con- fidence to the reliability of the analytical procedures applied to plasma and tissue containing high fluoride levels. These data indicate that a rapid equilibrium of fluoride between these tissues and the plasma occurred within 10 min after a large dose of fluoride was given intraperitoneally. This would imply that essentially all of the fluoride of bone had exchanged in a short period of time.

In the animals receiving the radiofluoride the highest concentrations of fluoride in plasma were attained by 30 min, and possibly as early as 10 min, and then decreased rapidly with time. This was not the time sequence for tendon, muscle or bone. The fluoride in tendon appeared to be high for at least 30 min and then decreased rapidly in the next 30 min, whereas the fluoride content of humeri and muscle peaked by 30 min and did not show any loss at 60 min.

Based on the specific activity of the injected fluoride solution, the concentration of fluoride in the entire body prior to receiving the dose and the amount of radioactivity in the muscle, the fluoride concentration in muscle 10 min after animals were given 3, 4 or 5 mg of fluoride was equal to approximately 20% of the fluoride concentration in plasma and was near the maximum level observed at the 60 min experimental period. By 30 min the concentration in the muscle was 30–35% of that in plasma. There was little change in this relation at 60 min for those animals receiving 3 or 4 mg of fluoride. However, those animals given the 5 mg dose had reached a concentration in muscle of approximately 50% of the fluoride level in plasma by 60 min. The muscle obviously attained a high fluoride concentration (7 and 9 ppm) by 10 min which was maintained for at least 60 min by those animals receiving 4 or 5 mg of fluoride respectively, whereas those animals receiving 3 mg of fluoride exhibited a sharp decline in concentration between 30 and 60 min from a maximum concentration of 5 ppm attained by 10 min.

Summary. Young male rats (~200 g) were employed to study the distribution of parenterally administered inert and radiolabeled fluoride. Animals receiving 25 mg of fluoride/kg body weight did not live longer than one hr and the plasma fluoride level reached a concentration of 48 ± 2.1 ppm. Other animals receiving 15 or 20 mg of fluoride/kg

had plasma fluoride levels greater than 30 ppm 10 min after the dose but returned to preexperimental levels (~0.10 ppm) by 24 hr. Total and ionic calcium levels in plasma were severely depressed in all animals following the fluoride administrations. The calcium levels at 30 and 60 min did not seem compatible with life although animals receiving 20 mg or less of fluoride/kg survived 24 hr. Radiofluoride concentrations in plasma, muscle, tendon, bone and gastrointestinal tract indicated the relative distribution and retention of the dose as well as the fluoride concentration at 10, 30, and 60 min after the dose was given. Muscle fluoride levels at all dose levels were 5–9 ppm after 10 min. These levels rapidly decreased in animals receiving 15 mg/kg but remained relatively constant for the other animals.

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;ynthesis of Insoluble Elastin in Hamster Lungs during Elastase-Emphysema (40055)

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itly, an experimental model of em- ı induced by elastase has been exten- udied (1–4). In the elastase model of ental emphysema, a single injection se caused diffuse destruction of lung ıl components and led to a lung mor- which resembled human panlobular :ma. It has been observed in this hat the elastin content of the lung :d shortly after the elastase instillation lually returned to a nearly normal ithin 60 days (5). The possibility of ıcement of this connective tissue fiber elastase-injury by active synthesis of ıas been indicated (5). This report s further studies of elastin synthesis ngs of hamsters with elastase-induced :ma.

re elastin has been considered a bio- ' stable and metabolically inert sub- 6). For example, in aortic tissues of rats, Slack (7), Kao *et al.* (8) and *et al.* (9) showed that elastin has no ırnover. Previous work in our labo- as shown that elastin in lung tissue is rt, with little or no synthetic activity rotein in the mature lungs of normal s (Yu, S.Y. and Keller, N.R., unpub- ata). Therefore, in mature hamsters, cult to label *in vivo* with a radioactive ›r by the conventional technique for ; the metabolism of elastin.

ollowing three procedures were used tudies of elastin synthesis.

he synthetic activity of lung elastin asured by comparing rates of incor- ı of [^{14}C]-L-proline into elastin be- he experimental and control lungs. C]-L-proline was administered by a ' injections which followed the elas- tase-instillation. (b) The synthetic activity of lung elastin was also studied in hamsters which had been labeled with a series of injections of [^{3}H]-L-proline during a rapid growth period and subsequently injected with elastase 3 months after the labeling. (c) Finally, since desmosines in elastin are characteristic amino acids and only found in fully cross-linked elastin, the rate of incorporation of [^{14}C]-L-lysine into desmosines found in lung extracts from the experimental and control group was compared.

Materials and methods. Incorporation of [^{14}C]-L-proline into lung elastin. All hamsters used in the experiments were obtained from Engle Laboratory Inc., Farmersberg, IN.

Experimental emphysema was developed in mature male hamsters, weighing 115–120 g each, by an intratracheal instillation of 0.3 ml of saline solution containing 0.25 mg or 25 Sachar's units of porcine pancreatic elastase (2). For controls, the animals were instilled with 0.3 ml of saline solution. Three days after the instillation of elastase, all hamsters, 25 controls and 21 experimentals, received a series of 14 intraperitoneal injections of [^{14}C]-L-proline, at intervals of 12 hrs; each dose containing 0.2 ml of 0.9% NaCl solution and 4 μCi of uniformly labeled [^{14}C]-L-proline, SpA 225 Ci per mole (New England Nuclear).

Preparation of insoluble elastin. Lungs were pooled respectively, homogenized with 100 vol of cold 0.9% NaCl, and then the insoluble residues were extracted in polyethylene plastic centrifuge tubes successively with the following solutions: 0.16 *M* NaCl solution at 4°, 5×; 5 *M* of guanidine · HCl, 0.1 *M* Tris pH 7.4, at 27°, 2×; 5 *M* of guanidine · HCl, 0.1 *M* Tris, 50 m*M* dithiothreitol and 0.1 *M* EDTA pH 7.4, at 27°, 2× (10); water, 3×; autoclaving at 15 lb for 4 hr, 2×; acetone, 3×; and ether, 2×. The insoluble residue was treated with 0.1 *M* NaOH at 98° for 45 min.

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The extracted residue (elastin) was washed several times with water, finally resuspended in water, lyophilized, and weighed. Dry elastin, 3–5 mg, was weighed on a filter paper and combusted in a Packard's Sample Oxidizer Model 306. Radioactivity was counted with a liquid scintillation counter, and expressed as specific activity.

Prelabelling Hamsters with [^{3}H]-L-Proline. Young male hamsters, litter mates, initially weighing 54 ± 5 g each, were chronically labelled with a series of intraperitoneal injections, one dose daily of 0.1 ml saline containing 2.8 μCi of [^{3}H]-3,4-L-proline (33.5 Ci/mmole, New England Nuclear), and the injections were repeated 22×. By the end of the labelling the animals weighed 88 ± 7 g and then were kept until body weight reached 117 g, which was approximately 3 months after the labelling. The solutions of elastase or saline were intratracheally instilled in the prelabelled hamsters, and lung elastin was prepared and radioactivity was measured as described previously (see above).

Incorporation of [^{14}C]-L-lysine into desmosines. Mature male hamsters, 18 of the elastase-instilled and 11 controls, were used. Three days after the elastase-instillation each animal received a series of 30 intraperitoneal injections, at 12-hr intervals, each dose containing 0.2 ml of saline and 1.1 μCi of uniformly labeled [^{14}C]-L-lysine, 279 mCi/mmole (New England Nuclear). The animals were sacrificed 18 days after the elastase-instillation. Lungs were pooled into control and experimental groups and extracted as described previously (see above), and the "autoclave" residue was obtained. The "autoclave" residue was hydrolyzed with 6 *N* HCl, and desmosines isolated. Desmosines in the hydrolyzate were separated by a preparative column of Aminex A-5 resin, 1.5 × 15.5 cm (Bio-Rad Lab), at 50°, by eluting with 0.15 *M* pH 4.5 pyridine–acetate buffer and 0.3 *M* pyridine (11). The corresponding fractions of isodesmosine and desmosine were collected, pooled, and the solvents evaporated. Each compound was further separated by an analytical column 0.9 × 13 cm with Beckman PA-35 resin and eluted at a flow rate of 68 ml/hr with sodium citrate buffers under the following conditions; at 55°, 0.2 *N*, pH 4.35 for 30 min, 0.2 *N*, pH 4.50 for 40 min and 0.38 *N*, pH 4.50 for 60 min. The fractions were collected, and aliquots of the appropriate fractions were used for determination of the amount of desmosines in the analytical column. A second aliquot was used for counting radioactivity by a liquid scintillation counter.

The elastase-instillation produced a distension of normal lung structure, which has been studied using the mean linear intercept method to quantify this dilation (2) (12). The present study yielded a similar change in the gross morphology of lungs of the elastase-instilled hamsters.

Results. Post-elastase labelling. Elastin synthesis, studied by the incorporation of [^{14}C]-L-proline in which the isotope was administered after the elastase-instillation, showed that the specific activity of elastin in the lungs of the elastase-instilled groups increased 8–14 fold over those of the respective control (Table I). Within the experimental group, there was a reduction of the specific activity in lung elastin of day 30 compared to that of day 18 which presumably was a result of the dilution of the radioactive elastin by nonradioactive elastin synthesized during the period of days 18 and 30. These data suggested that elastin synthesis was active, not only in the period 3 to 18 days after the elastase-instillation but also during the period 18 to 30 days.

Preelastase labelling. Elastin synthesis, studied in the prelabelled hamsters, showed that the specific activities of lung elastin were lower in the elastase-instilled groups as compared to those of controls (Table II). Since

TABLE I. INCORPORATION OF [^{14}C]-PROLINE INTO LUNG ELASTIN OF CONTROL AND ELASTASE-INSTILLED HAMSTERS.[a]

	Sp Act [^{14}C] dpm/mg	Average	Rate of incorporation
Control day 18	165[b]		1
Control day 30	208[b]	$\bar{x}$ = 187	1
Elastase day 18	2552[b]		14
Elastase day 30	1429[b]		8

[a] Hamsters were intratracheally instilled with the elastase, and then a series of [^{14}C]-L-proline injections was carried out. Control groups at days 18 and 30 contained 15 and 10 hamsters respectively, while the elastase-instilled groups contained 11 and 10 hamsters.

[b] Each experiment was based on at least two determinations of the radioactivity, and the average was obtained.

: II. SPECIFIC ACTIVITIES OF LUNG ELASTIN
M PRELABELED HAMSTERS, CONTROL AND
ELASTASE-INSTILLED.[a]

	Sp Act ^3H dpm/mg	P^c	% decrease[b]
day 18	640 ± 48[b]		
day 30	662 ± 50[b]		
day 18	561 ± 62[b]	NS	14%
day 30	429 ± 62[b]	$P < 0.05$	34%

ısters were prelabeled with [^{3}H]-L-proline during growth period and then 3 months after the instilled with the elastase intratracheally. The were sacrificed 18 and 30 days after the elastase, control groups containing five animals and the ental groups containing four animals.

n ± SEM. The data were compiled from three ents.

ıpared with the corresponding control; NS, not nt.

ıpared with the average of control day 18 and

:-normal lung showed no active syn- of elastin, a decrease in the specific y of the prelabelled elastin by incor- ɔn of non-radioactive elastin after the e-instillation indicated the synthetic y of elastin. This decrease in the spe- tivity after the elastase-instillation was ed in lung elastin of day 30 ($P < 0.05$).

l-L-lysine incorporation into desmosines. ıgh the insoluble elastin as prepared d the characteristic amino acid com- ın with no evidence of possible contam- ı by other impurities (5), this method ı subjected to criticism since the elastin ed is the insoluble residue after the itive extractions which include hot 0.1)H. Thus the possibility of losing elas- this treatment or of contamination by insoluble materials might remain, es- y in the case of extracting tissues of ɔd, aged or extremely young animals. ver, studies using desmosines, which ıino acids exclusively found in mature- ble elastin, should nevertheless provide ight into the metabolism of insoluble . The result of the incorporation of -lysine into desmosines is presented in III. The specific activities of desmo- isolated from the lung fractions ob- from the elastase-instilled hamsters ıgher than those of the corresponding ls, 3600 and 3800 dpm per μmole of nosine and desmosine, vs 309 and 190 f isodesmosine and desmosine, for the experimentals and control, respectively.

Discussion. In lungs of the hamsters with elastase-induced emphysema, there was a decrease in the amount of elastin shortly after the injection of elastase, presumably resulting from proteolysis by the enzyme (Table IV); thereafter, the amount of elastin per lung rose and then became stable within 18 days after the elastase-injection. In addition to this active elastin synthesis in lungs of the elastase-instilled hamsters, the accelerated turnover of elastin persisted beyond the point of the restoration of nearly-normal amounts of elastin.

This activation of elastin synthesis after lung injury by elastase was accompanied by simultaneous collagen synthesis (13), therefore we believe that this activation of mesenchymal tissue resulted from a tissue response to the specific injury to the lung. The synthesis of elastin which was observed in hamster lungs after the elastase-instillation is

TABLE III. INCORPORATION OF [^{14}C]-L-LYSINE INTO DESMOSINES IN AUTOCLAVE RESIDUES OF HAMSTER LUNGS, CONTROL AND ELASTASE-INSTILLED.[a]

	No. of lungs	Isodesmosine[b] dpm/μmole	Desmosine[b] dpm/μmole
Control	11	309	190
Elastase	18	3808	3636

[a] Hamsters were intratracheally instilled with the elastase solution and then a series of [^{14}C]-L-lysine injections was carried out. The animals were sacrificed 18 days after the elastase-instillation, and then the lungs were pooled. The autoclave residues were prepared after a series of extractions with solutions of NaCl, guanidine · HCl and autoclaving.

[b] Desmosines were obtained from hydrolysates of autoclave residues of hamster lungs after purification on ion exchange columns. The amount of desmosines were estimated by the ninhydrin reaction using lysine as the standard.

TABLE IV. ELASTIN AND TOTAL DESMOSINES IN HAMSTER LUNGS OF CONTROL AND ELASTASE-INSTILLED.

	Days after Injection[a]	Desmosines μmole/lung	Elastin[b] mg/lung
Control	3 (2)	0.251	2.1
Elastase	3 (2)	0.092	0.8
	18 (2)	0.221	1.8
	30 (2)	0.245	1.9

[a] Numbers in parentheses indicate number of animals used.

[b] The estimation was based on the concentrations of 0.070 μmole desmosine and 0.055 μmole isodesmosine per mg elastin.

seemingly a reparative process in response to the elastase-injury.

Kuhn and Tavassoli (14) observed from morphological evidence that the rearrangement of the lung structure had been continuing up to 2 months after the elastase-instillation in similar experiments. This suggests the possibility that the synthesis and degradation of elastin might be activated and persisted for a long time in these lungs. There is some evidence from our data (Table II) which suggested that the synthetic activity in lungs remained elevated even 18 days after the elastase-instillation, although it is not certain whether the elastin synthesis continues beyond one month after the elastase-instillation. If the synthesis of elastin were continuing in lungs of the hamsters with elastase-induced emphysema, the degradation of elastin might have occurred to balance out the increase.

The insoluble elastin, prepared by the present method, probably was "pure" lung elastin and contained little or no contaminating substances such as tropoelastin (15) or microfibril (10). The presence of the latter compound was negligible because the elastin prepared contained no significant amount of polar amino acids. However, despite the anatomically distinguishable appearance of lung elastic fibers in the elastase-instilled hamsters (5) (12), our studies failed to show any significant change in the amino acid composition of lung elastin obtained from hamsters 4 months after the elastase-instillation. This might be due to the hot alkaline treatment *per se* since Sandberg pointed out that elastin prepared from different tissues and species by this method showed similar amino acid composition because the hydrolytic nature of the reagent reduced elastic fiber to a common composition (16). Therefore, the possibility of the presence of a different elastic tissue in the emphysematous lungs might still remain.

In the lung, elastin occurs in airways, vascular trees, alveolar septa, and pleura. It is, however, impossible to identify the exact site of the synthesis by the present method, despite the demonstration of elastin synthesis in lungs of the hamsters with elastase-induced emphysema. By morphological observations (12) (17), the site of the elastase-injury was observed more frequently in perivascular areas, alveolar septa, and pleura; however, discrete and fibrillar elastic fibers were often found in alveolous septa, and a spongelike elastic structure was seen in peripheral areas of the elastic lamella under the mesothelial plane. Although these elastic fibers were observed only in lungs of hamsters with the elastase-induced emphysema, it is not known whether these fibers are some of the degradated products resulting from the elastase-instillation or newly synthesized fibers.

Following an injury, the normal tissue response is to repair by synthesizing collagenous fibers but not elastic fibers. The reason for the synthesis of both elastic and collagenous fibers in the emphysematous lungs is not known, although a similar situation was also observed in cultured aortic cells which formed both collagen and elastin (18–20).

Summary. The synthesis of elastin *in vivo* in normal-mature animals is difficult to demonstrate because of a very slow turnover of this protein. Thus most of the studies of elastin synthesis have been limited to aortic tissue cultures or in *in vivo* young animals. Our data indicate that the lung, upon elastase-injury, synthesized elastin at demonstratively faster rates than normal. However, the mechanism by which the activation of elastin synthesis occurs in the emphysematous lungs remains for further study.

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Hypocalcemia and Increased Serum Calcitonin in Baby Rats Given Glucose Orally[1,2] (40056)

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Recently, we reported that 14-day-old suckling rat pups exhibit high levels of circulating immunoreactive calcitonin (CT) (1). In normocalcemic pups suckling *ad lib.*, serum CT levels as high as 4000–5000 pg/ml were observed. If the babies were fasted 12 hr or longer, serum CT fell to <150 pg/ml. When babies fasted for 12 hr were allowed to suckle again, both serum calcium and serum CT rose at 30 and 60 min; by 90 min serum calcium had returned to normal but serum CT remained high (1).

Because serum CT was high in normocalcemic suckling baby rats, we hypothesized that, in addition to a rise in blood calcium, some gastrointestinal factor might be involved in stimulating the baby rat to secrete CT during ingestion and absorption of milk (1). As a first approach to testing this hypothesis, we decided to orally administer a calcium-free solution of glucose to rat pups and see whether or not increased CT release occurred. Under these conditions, any increase in CT could not be secondary to a rise in serum calcium during absorption of calcium from the gut.

Materials and methods. Animals. Female Zivic-Miller rats (Zivic-Miller Co., Allison Park, PA), with litters of eight to ten pups each were received in the laboratory when the babies were 6 days old. Experiments were performed when the babies were 15 days old.

Procedures and analyses. All babies were separated from the mother 13–16 hr (overnight) before each experiment. Half of the babies in each litter were assigned to different treatment groups. Babies given glucose were administered 1.5 ml isotonic (5% w/v) glucose in distilled water. Control babies received either no solution or 1.5 ml isotonic (0.9% w/v) NaCl. Solutions were delivered directly into the stomach by gavage using a marked length of Intramedic polyethylene tubing (PE50, inner diameter 0.023 in.) attached to a syringe. Babies were lightly anesthetized with ether during the gavage procedure.

Thyroparathyroidectomy was performed by blunt dissection under ether anesthesia. Sham operation involved simply exposure of the thyroparathyroid complex.

At the desired time interval after treatment, blood was obtained from each baby under ether anesthesia. In selected experiments the tip of the tail was severed and blood was collected in a heparinized microhematocrit tube. After centrifugation of the tubes for 2 min in a Model MB microhematocrit centrifuge (International Equipment Co., Needham Heights, MA), hematocrit values were read using a Model CR microhematocrit tube reader (International Equipment Co.).

Blood also was obtained from each baby by cardiac puncture using a 27 gauge, ½ in. needle. Serum was collected by centrifugation within 1 hr of blood collection, and each sample was subdivided, one portion being analyzed freshly for serum calcium and inorganic phosphorus and the other being stored at −20° until subjected to radioimmunoassay. Both calcium (2) and inorganic phosphorus (3) were analyzed by automated procedures using an Autoanalyzer (Technicon, Inc.).

CT in serum samples was measured by radioimmunoassay using an homologous system for rat CT. The procedure has been described earlier in detail and both intra- and interassay variations have been reported (4–6).

Glucose in serum was analyzed using a colorimetric procedure (7).

Statistical analyses. For serum calcium, phosphorus, glucose and hematocrit, data

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from individual experiments were subjected to analysis of variance (8), and standard errors were calculated from the residual error term of the appropriate analysis. Significance of differences between groups was determined using either the F test or the multiple comparisons test of Hartley (8).

In one experiment where all values for CT were detectable (Fig. 3), data were subjected to analysis of variance and comparisons made as just described. In other experiments where some values for CT were below the limits of detectability of the immunoassay (Fig. 2) mean values could not be calculated. Therefore, individual values are shown and the significance of differences between groups was evaluated using the nonparametric test of Wilcoxon (8).

In Table I and Figs. 1 and 2, results of identical experiments conducted on separate days were combined because the variances were homogeneous and statistical analyses showed that the results were not significantly different.

Results. Figure 1 and Table I (Groups A and B) summarize the combined results of six separate experiments which showed that 1.5 ml of 5% glucose given orally by gavage to thyroid-intact baby rats produced a significant fall in serum calcium 60 min later. The fall in serum calcium was accompanied by a fall in serum phosphorus as well (Fig. 1). No decrease in either serum calcium or serum phosphorus occurred when the same volume

TABLE I. EFFECT OF ORAL GLUCOSE ON SERUM CALCIUM IN ACUTELY THYROIDECTOMIZED AND SHAM OPERATED BABY RATS.

Treatment	No.	Serum Ca (mg/dl)	P^b
A. SHAM + NaCl p.o.	15	10.2 ± 0.12	<0.001
B. SHAM + Glucose p.o.	14	9.5 ± 0.13	
C. TPTX + NaCl p.o.	14	9.8 ± 0.13	N.S. (>0.1)
D. TPTX + Glucose p.o.	13	9.3 ± 0.14	

[a] Baby rats, 15-days old, were fasted overnight before surgery (see Methods). Immediately after surgery (sham op. or thyroidectomy by thyroparathyroidectomy), babies were given 1.5 ml of 0.9% NaCl or 5% Glucose by gavage. Blood was collected 1 hr after gavage.

[b] Values are presented as mean ±SE and represent the combined results from three separate experiments.

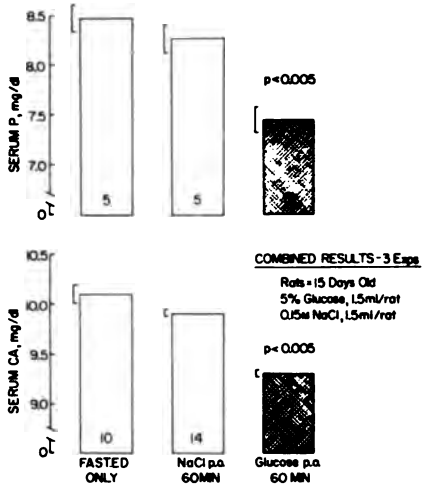

FIG. 1. Hypocalcemia and hypophosphatemia in baby rats given glucose orally. Babies were fasted overnight before the experiment and either simply bled (Fasted Only) or given NaCl or glucose as indicated and bled 1 hr later. Each bar represents the mean value for the number of babies shown within the bar, and the brackets show the SE.

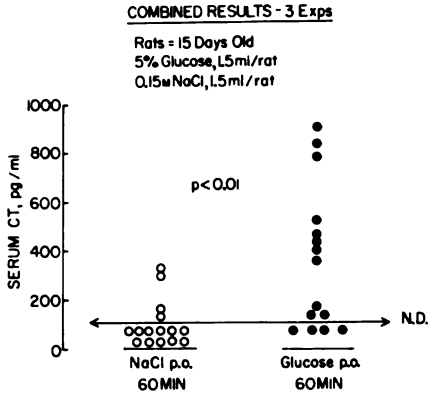

FIG. 2. Increased serum CT in baby rats given glucose orally. Babies were fasted overnight before the experiment, given NaCl or glucose as indicated and bled 1 hr later. Each point represents an individual baby, and the horizontal arrow shows the lower limit of detectability of the radioimmunoassay for rat CT (N.D. = Not Detectable).

of isotonic saline was administered (Fig. 1, saline vs. fasted only).

The fall in serum calcium which occurred in baby rats 60 min after oral glucose was accompanied by a significant increase in immunoreactive serum CT (Fig. 2).

The results suggested that the fall in serum calcium after glucose might, at least in part,

be due to the increase observed in circulating CT. To test this idea glucose was administered to baby rats with intact thyroid glands and to babies deprived of endogenous CT by acute thyroidectomy (Table I). The results showed that glucose produced a significant fall in thyroid intact pups but not in the babies subjected to thyroidectomy. A small decrease in the mean value for serum Ca (C vs D) was apparent in the thyroidectomized pups given glucose in each of three experiments but it did not reach statistical significance in any of them ($P > 0.1$). Likewise, a small but insignificant fall in the mean serum calcium was observed after acute thyroid removal by thyroparathyroidectomy (A vs C) which probably can be attributed to the loss of the parathyroid glands for 1 hr.

In order to examine the time course of the changes in serum calcium and CT after glucose, the experiment illustrated in Fig. 3 was conducted. The results showed that serum CT was significantly elevated and serum calcium was significantly decreased only at 60 min after glucose. Values had not changed at 30 min after glucose and had returned to control levels by 90 min. In contrast, the serum glucose was highest at the earliest time period examined (30 min) and had declined toward the control level by 60 and 90 min. As observed in this experiment (Fig. 3), as well as in several others not shown, no change in plasma hematocrit was observed after administration of glucose.

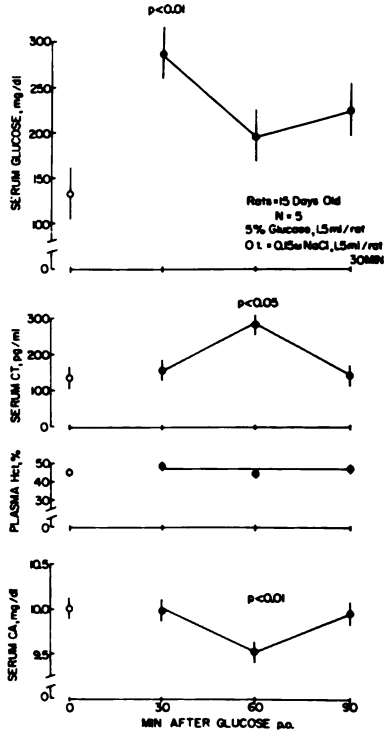

FIG. 3. Time course of increase in serum CT and decrease in serum calcium in baby rats given glucose orally. Babies were fasted overnight, given glucose as indicated and bled 30, 60 or 90 min later. Values for 0 time represent fasted babies given NaCl orally and bled 30 min later. Each point represents the mean value for a separate group of five pups, and the vertical line shows the SE.

Discussion. These experiments were designed to determine whether or not a calcium-free solution of glucose given orally could increase release of CT in the baby rat. The results clearly show that serum CT was elevated 60 min after a gavage with isotonic glucose in modest amounts (Fig. 2). The increase in CT was accompanied by a fall in both serum Ca (Figs. 1 and 3 and Table I) and serum phosphorus (Fig. 1). The changes induced by glucose were not nonspecific, e.g., simply due to gastrointestinal distension, because control gavages with saline were without effect (Fig. 1).

The glucose-induced decrease in serum calcium probably was, at least in part, due to the increase in serum CT. This conclusion is based on the observation that glucose produced a significant decrease in serum calcium only in the presence of the thyroid gland and endogenous CT (Table I).

The decrease in serum phosphorus after glucose also may partly be produced by CT which is well known to cause hypophosphatemia as well as hypocalcemia. However, it has long been known that hypophosphatemia results from glucose administration and this has been attributed to phosphorylation and cellular uptake of glucose (9). Hypophosphatemia also occurs together with hypoglycemia when young adult rats are injected with insulin (10). We have routinely observed significant ($P < 0.01$) hypophosphatemia 30 min after injection of 5 units of insulin iv in 35-day-old rats. This high dose of insulin, which lowers serum glucose from ~120 mg/dl to ~30 mg/dl ($P < 0.001$) does not lower serum calcium. Since glucose does lower se-

alcium in these young adult rats ($P <$ ust as it does in the babies but insulin ıot, it seems unlikely that insulin me- the increase in CT and decrease in calcium observed after oral glucose. the present results show that the es in CT and calcium in blood are not lary to changes in plasma volume, since ocrit was unaffected by oral glucose. mechanisms by which oral glucose es blood CT and lowers serum calcium ı to be elucidated. However, our find- o agree with the observations reported ynen and Franchimont (11) that, in CT increased after an oral glucose tol- test at a time when the concentration od sugar was falling.

think that some as yet unidentified intestinal factor or hormone may well olved in the CT response of baby rats kling and to oral glucose. Although ı and its structural analogs, e.g. CCK, otent CT secretagogues in the pig) and man (15), they are weak or in- ve in causing release of rat CT (5, 16). r studies will be required to pursue and y the factor or events involved in CT during feeding and absorption of milk uents in the baby rat. However, our t results show clearly that even simple components of ingested milk may con- part of the stimulus for CT secretion.

mary. Baby rats, 15 days old, were >13 hr and given 1.5 ml 5% glucose by h tube. Control babies were gavaged .5 ml 0.9% NaCl which itself did not serum calcium or CT.

age with glucose produced a fall in calcium of 0.5–1.0 mg/dl 60 min later).05–<0.005). Glucose produced a fall m phosphorus at 60 min similar to that ım calcium. Measurement of immuno- e CT in serum showed a significant om ~120 pg/ml or less up to as much pg/ml ($P < 0.01$). A time course study l that serum CT increased and serum n decreased only at 60 min after oral ; values had not changed at 30 min d returned to control levels by 90 min. In contrast, serum glucose was high (~300 mg/dl) at 30 min after gavage and had returned toward the control level (~130 mg/dl) by 60–90 min. The decrease in Ca after glucose must, at least in part, be due to CT since after thyroidectomy glucose did not produce a significant fall in serum calcium.

The findings show that oral administration of a calcium-free solution can increase CT release in the absence of a rise in blood calcium.

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Circulating Interferon in Sudden Infant Death Syndrome (40057)

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Although the etiology of Sudden Infant Death Syndrome (SIDS) is unclear, its epidemiology strongly suggests that viral infections may play a significant role (1). Attempts to demonstrate the etiologic importance of viruses have been directed towards direct viral isolation from SIDS cases with recovery rates varying up to 37.5% (2, 3). This variation has partly been attributed to the different methods used for specimen processing and isolation.

Since circulating interferon has been detected in infections produced by live virus vaccines (4), in patients with naturally occurring viral infections (5) with some studies showing a correlation between circulating interferon and viremia (6), another suggested approach of establishing the occurrence of viral infections in SIDS is indirectly through the detection of circulating interferon (7).

This report describes our attempts to search for evidence of viral infections in SIDS by detecting the presence of circulating interferon.

Materials and methods. Study Population. Specimens were obtained from 56 infants who were autopsied at the Office of the Chief Medical Examiner, Baltimore, MD, during the 13-month period between January 1967 and February 1968. Forty-four infants were classified as SIDS by fulfilling the definition proposed at the Second International Conference on Sudden Death Syndrome of Infancy in that their deaths were unexpected by history and unexplained by the postmortem examination (8). This group included 20 females and 24 males from 6 weeks to 12 months of age with 37 cases being under 6 months. Specimens collected included tissue from lung, trachea, heart, brainstem, cortex, and serum from heart blood samples. These were frozen at −70° until used. In several instances the blood samples were unclotted.

In the control group of 12 infants with explainable deaths, there were six males and six females between 4 weeks and 14 months. The causes of death were attributed to trauma, burns, asphyxia, drowning, meningitis with adrenal hemorrhage, central nervous system malformation and hydrocephaly, trisomy 13–15, congenital heart disease, and pneumonia.

A second control group was included consisting of 10 adults between 24 and 74 years of age whose deaths were either caused by arteriosclerotic cardiovascular disease (ASCVD) or by gun shot or knife wounds (homicide). Despite the difference in age, inclusion of this group was thought to be necessary because of the insufficient numbers of traumatic and natural deaths in the infant control group.

Interferon Assay. Serial twofold dilutions of sera were made in Eagle's minimal medium (MEM) supplemented with 3% fetal calf serum (FCS). One ml of the dilutions was added in triplicate to confluent monolayer cultures of human diploid fibroblast (Wi-38) cells prepared in 16 × 125 mm screw-capped tubes. After 18 hours of incubation at 37°, the diluted sera were aspirated, and the cells were challenged with 100 $TCID_{50}$ of Sindbis virus. The cultures were maintained at 37° and read in 72 hr when the virus controls showed 100% (4+) cytopathic effect (CPE). The result was expressed in units/ml as the reciprocal of the dilution which reduced CPE by 50% (2+), as compared to the virus control.

Virus Isolation. Specimens of lung, trachea, heart, cortex, and brainstem were made into 10% suspensions by dilution and grinding in an appropriate volume of MEM. Attempts at virus isolation were limited to only those cases in which circulating interferon was detected. Aliquots of tissue suspension were inoculated into duplicate screw cap tubes

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ıining monolayer cultures of human am-
, human embryonic kidney, and Wi-38
The cultures were maintained at 37°
observed daily for CPE. After 14 days,
:ells were scraped from the walls of the
tive cultures, and the cell suspension
as inocula for new cultures that were in
observed for 2 weeks. Hemadsorption
tested for by using a 1% suspension of
:a pig erythrocytes.

:sults. A viral inhibitor was detected in
erum of 3 cases of SIDS and of 1 control
le I). This inhibitor had properties char-
istic of interferon. It was species specific,
lialyzable, trypsin sensitive, stable at
was not sedimented by ultracentrifu-
ın at 110,000*g* for 3 hr, and did not
tly inactivate the challenge virus.

ıaracteristics of the infants in whom in-
ron was detected are listed in Table II.
interferon titers in units/ml in the three
: of SIDS were 32–64, in comparison to
:r of eight in the one control case. Virus
.res were negative in all four cases. The
ological findings in the three SIDS cases
negative, as expected, but in the control
the findings included pneumonia, sub-
l hematoma, and bilateral otitis media
middle ear cultures growing Klebsiella–
bacter bacteria.

scussion. Our results show that circulating interferon is not frequently detected in SIDS. Interferon was found in only three of 44 cases of SIDS in comparison to one of 22 control cases with the frequency and titers not being significantly different. The finding of interferon in the infant with pneumonia and otitis media with middle ear cultures positive for Klebsiella–Aerobacter bacteria is not surprising since circulating interferon has been detected in patients with gram negative infections (9).

Our data could be interpreted in several ways. The infrequent finding of circulating interferon could be due to too early sampling before there was an interferon response, to an insensitive assay system, or to inactivation of the interferon by proteolytic enzymes released at death. Arguments against this include the observation that interferon usually appears in serum early in the course of infection at the time that viremia occurs (6), the use of a similar type of interferon assay as reported here in detecting circulating interferon in children with acute respiratory infections (5), and studies showing that there is an accurate correlation in antemortem and postmortem measurements of serum proteins such as globulins and albumin (10).

The results could best be interpreted to suggest that virus infections in SIDS are not of a disseminated type since circulating interferon was not frequently detected. This is consistent with the findings in most studies involving viral isolation in SIDS where recovery of viruses has been limited to the respiratory and gastrointestinal tracts (3). Our studies are similar to those previously reported in which attempts were made to detect viremia in SIDS by measuring circulating interferon (7). They also support the hypothesis that if viruses do play a role in the final

ABLE I. CIRCULATING INTERFERON IN SIDS.

Group	Number	Number with interferon
SIDS	44	3
*Control	12	1
**Control	10	0

Infants with known causes of death.
Adult death due to homicide or arteriosclerotic cardiovascular disease (ASCVD).

TABLE II. INFANTS WITH CIRCULATING INTERFERON.

ıse	Interferon Titer	Age	Sex	Month of Death	Virology Cultures	Pathology
	64	6 months	Male	April	—	—
	64	5 months	Male	December	—	—
	32	6 weeks	Female	January	—	—
rol	8	2 months	Female	February	—	Pneumonia, subdural hematoma, bilateral otitis media with middle ear culturing Klebsiella—Aerobacter bacteria

Expression in units/ml as the reciprocal of the dilution of sera which reduced CPE by 50% (2+) as compared : virus control.

common pathway of SIDS, they may possibly function in a local manner (8), the mechanism of which remains to be determined.

Summary. Circulating interferon, which may be indirect evidence of viral infections, was searched for in the Sudden Infant Death Syndrome (SIDS). A viral inhibitor with the properties of inteferon was detected in the serum of three out of 44 cases of SIDS, and in one out of 22 control cases. These results show that circulating interferon is not frequently detected in SIDS, and suggest that if viruses do play a role in the final common pathway of SIDS, they may possibly function in a local manner, rather than in a disseminated type of infection.

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erential Depression of IgM Producing Immunocytes during Mastocytoma-induced Immunosuppression (40058)

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stocytoma cells derived initially from cells of DBA/2 mice are markedly im- suppressive. Immunologic impairment when relatively small numbers of these cells are added to normal spleen cells nized *in vitro* with sheep red blood cells C) or alloantigens (1, 2). Cell-free ex- and/or ascites fluid from tumor-bear- nimals also are immunosuppressive *In vivo* studies have shown that im- logic impairment occurs in mastocy- bearing mice in the late stages of tumor ssion (6). However, early after implan- of mastocytoma cells there is no signif- suppression of immune responsiveness metimes even an enhancement.

ibody formation to SRBC in mastocy- bearing mice, as well as in spleen cell es treated with mastocytoma cells or ts, has been measured only at the level IgM production. Since it is possible he tumor cells may affect differently nocytes involved in a primary vs. sec- y immune response it seemed of value ermine the effects of mastocytoma on antibody producing cells. In the pres- dy the effects of the immunosuppres- astocytoma cells and cell-free extracts pearance of IgM vs. IgG antibody pro- cells after primary or secondary im- ation were examined.

hods and materials. Animals. Male 2 mice obtained from Cumberland Farms, Clinton, TN, were used for these ments. The animals were approxi- 6–8 weeks of age and weighed 18–20 n used. They were fed Purina mouse and water *ad libitum.*

nor cells. Mastocytoma tumor cells, des- d PB815X, were obtained originally he American Type Culture Collection, ille, MD. They were passaged through 2 mice by intraperitoneal (ip) injection other week.

gen. SRBC were obtained in Alsever's solution from Cappel Laboratories, Downingtown, PA, and washed several times with minimum essential medium (MEM) purchased from Microbiological Associates, Bethesda, MD. Mice were injected by intravenous (iv) inoculation of 0.2 ml of a 10% suspension of freshly washed erythrocytes.

Antibody assay. Both the direct and indirect assays for hemolytic plaque forming cells (PFCs) to the SRBCs were performed with the spleen cell suspensions essentially as described earlier (1, 3). For the direct assay varying numbers of washed spleen cells from control and mastocytoma-bearing and/or ascites fluid injected mice were tested by the localized hemolytic gel assay essentially as described initially by Jerne *et al.* (1, 3). For this purpose 0.1 ml of a cell suspension was added to 2.0 ml melted Difco agar (40°) containing an 0.1 ml inoculum of a 1% suspension of RBCs. The agar cell mixture was poured carefully onto the surface of a previously prepared 60 mm diameter Petri plate containing a base layer of solidified agar. After the upper layer solidified the plates were incubated for 1 hr at 37° and then treated with approximately 5 ml of a 1:15 dilution of sterile guinea pig serum as the source of complement. The plates were incubated further for one to 2 hr until zones of hemolysis developed. These were considered due to direct 19S IgM PFCs. For indirect assay a facilitation procedure was utilized with antimouse gamma globulin serum (7, 8). To each agar plate was added 0.1 ml of a 1:100 or 1:200 dilution of rabbit antimouse 7S IgG serum with specificity to the heavy chain. The additional PFCs which developed on such plates were considered due to 7S IgG antibody (i.e., the numbers of direct PFCs were subtracted from the total number after the additional plaques appeared). In all cases the numbers of anti SRBC PFCs were determined on duplicate plates containing spleen cells from a single animal and the average

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number of PFCs was calculated for each mouse spleen.

Ascites fluid. Mice were injected by ip inoculation of 10^6 tumor cells. Fourteen to fifteen days later the ascites fluid (AF) was obtained from individual mice by aspiration of the peritoneum with a needle and syringe. Pooled AF was clarified by centrifugation at 2000*g* at 4°. The cell-free supernatants were frozen in small aliquots at −20°. The AF was thawed and warmed to 37° immediately before ip injection into test mice.

Results. Mice bearing mastocytoma tumors showed significant suppression of the IgM PFC response after either primary or secondary immunization with SRBC (Table I). For example, whereas normal mice developed many splenic IgM PFCs, with peak numbers on day 5 after immunization, mice given 10^6 mastocytoma cells ip 15 days or longer before primary immunization showed a depressed response. This suppression was evident on each day of assay. Although the peak response was about two thirds lower than that occurring in nontumor bearing control mice, the day of peak response still occurred on the same day, i.e., day five.

The indirect 7S IgG PFCs appeared at a relatively slower rate in both normal and tumor-bearing animals after primary immunization, with the peak on days 6 to 8. At these times, however, there were two to three times as many IgG as compared to IgM PFCs. The mastocytoma-bearing mice had essentially the same number of 7S IgG PFCs as did control mice. During the secondary immune response there was also no significant depression of the IgG PFC response (Table I). For these experiments mice were primed with sheep RBCs and injected with mastocytoma cells 15 days later. All mice received a second injection of sheep erythrocytes one month after the first injection. There was a marked depression of appearance of IgM PFCs in the spleen of these animals (approximately 75% fewer PFCs on the peak day as compared to control animals). In contrast, little if any effect on the IgG PFC response was evident in the tumor bearing animals (Table I). In other experiments, not shown, little if any effect was evident on the IgG PFC response in mice primed with SRBC at different times prior to injection of mastocytoma cells and secondary immunization.

Since it was difficult to control the size of the tumors in the mice at various times after implantation of mastocytoma cells, and since the absolute numbers of tumor cells per mouse was variable, addtional experiments were carried out in which known amounts of AF harvested from tumor-bearing mice were injected prior to primary or secondary immunization with SRBC. As can be seen in Table II, mice injected with 0.5 ml AF before primary challenge immunization with SRBC showed a marked impairment of their expected PFC response. However, this suppression was evident mainly at the level of IgM PFCs. The appearance of 7S IgG PFCs was

TABLE I. HEMOLYTIC ANTIBODY PLAQUE RESPONSE IN THE SPLEEN OF CONTROL AND MASTOCYTOMA-BEARING MICE AFTER PRIMARY VS. SECONDARY IMMUNIZATION WITH SRBC.

Anti-SRBC response on day after immunization[a]	Mouse group tested			
	Normal controls		Mastocytoma injected[b]	
	IgM PFCs	IgG PFCs[c]	IgM PFCs	IgG PFCs[c]
Primary +4 days	64,500	13,600	12,300	15,700
+5	97,300	58,960	38,700	65,400
+6	71,500	165,000	31,500	150,000
+8	40,350	130,500	22,300	125,000
Secondary +3	9,750	73,100	1,500	46,500
+4	12,600	195,000	3,750	170,000
+5	11,700	210,000	2,130	195,000
+7	5,650	97,500	1,600	112,000

[a] Groups of five to eight mice given either one or two iv injections of 4×10^8 SRBCs; secondary injected mice given booster immunization 30 days after primary injection.

[b] Mice injected ip with 10^6 mastocytoma cells 14 days before either primary or secondary immunization.

[c] Indirect 7S IgG PFCs detected by facilitation procedure with antiglobulin serum.

E II. CYTOKINETICS OF APPEARANCE OF HEMOLYTIC PFCs IN THE SPLEEN DURING PRIMARY RESPONSE RBCs BY NORMAL MICE AND MICE INJECTED ip WITH ASCITES FLUID FROM MASTOCYTOMA-BEARING MICE.

	Mouse group tested			
	Normal controls		AF injected[b]	
in days after pri- immunization[a]	IgM PFCs[c]	IgG PFCs[d]	IgM PFCs[c]	IgG PFCs[d]
0	<100	<100	<100	<100
−3	11,920	3,436	8,760	2,278
−5	86,750	29,750	15,750	21,500
−7	59,340	187,300	10,300	174,000
−10	21,150	145,500	8,450	130,000
−15	7,300	37,400	2,150	35,300

roups of five to six mice injected iv with 4×10^8 SRBCs and assayed on day indicated for PFCs.
lice treated by ip injection of 0.5 ml A.F. on day −1 and 0 relative to day of immunization with SRBC.
irect PFCs for high efficiency 19S hemagglutinin antibody.
ıdirect PFCs detected by facilitation procedure with antiglobulin serum.

tially normal in these AF-treated mice le II). The day of peak response of both s of PFCs was essentially the same, dless of whether or not the mice were d with AF.

rmal mice primed with SRBC and then a second injection of erythrocytes de- ed a markedly enhanced IgG PFC re- e. As is evident in Table III, mice in- l prior to secondary immunization with howed a depression of the IgM but not gG PFC response, similar to the sup- ion occurring in mice bearing mastocy- tumors. During the secondary resonse ol mice showed a rapid appearance of PFCs, with peak numbers 4 days after er stimulation. At this time there were t 25 times as many IgG PFCs as IgM in the control animals. Mice injected AF before secondary immunization ed esentially a similar IgG PFC re- e, but only about one third the IgM nse, as compared to controls. Thus on eak day of the secondary response there about 80 times more IgG PFCs than PFCs in AF treated mice. On days 6 to hen the numbers of IgM PFCs were asing rapidly, the numbers of IgG PFCs ined relatively elevated in the spleen of control and AF-treated mice.

cussion and conclusion. The results of experiments showed that mastocytoma- ng mice depressed in their ability to ss a normal IgM antibody response to C are, nevertheless, capable of a normal gG PFC response. Similar results were ned for both the primary and secondary antibody response. Mice given a single injection of SRBC showed a rapid appearance of specific IgM hemolytic PFCs, with peak numbers on days 4 to 5 after immunization. At this time some IgG PFCs were evident, especially since the mice were given an optimum dose of RBC. The peak IgG PFC response occurred several days later. Mice actively bearing a mastocytoma tumor showed a marked depression of the IgM response, but only a slight, if any, suppression of the IgG PFC response. This was evident when mice were inoculated with 10^6 tumor cells approximately 2 weeks before primary immunization.

When RBC primed mice were given mastocytoma cells two weeks before a secondary immunization, there was a similar difference in the IgM vs. IgG antibody response. The major difference between primary and secondary responses was the appearance of large numbers of IgG PFCs, with peak responses several days earlier than during the primary response. Mice injected with mastocytoma cells two weeks before secondary immunization had a marked depression of the 19S IgM antibody response, but no consistent alteration of response, despite the fact peak days were similar to controls.

In order to investigate whether suppression was due to the immunosuppressive factor(s) found in previous studies, AF from mastocytoma-bearing mice was injected into normal animals prior to primary or secondary immunization with SRBC. Similar to the findings with the mastocytoma-bearing mice, AF treatment resulted in a preferential sup-

TABLE III. CYTOKINETICS OF THE SECONDARY HEMOLYTIC PFC RESPONSE IN THE SPLEEN OF NOR MICE AND MICE INJECTED WITH A.F. FROM MASTOCYTOMA-BEARING MICE IMMEDIATELY BEFORE A B INJECTION WITH SHEEP RBCs.

Time in days after secondary immunization[a]	Mouse group tested: Normal controls IgM PFCs	Normal controls IgG PFCs[c]	AF injected[b] IgM PFCs	AF injected[b] IgG PF
0	1,250	650	975	59
+2	5,970	21,350	2,360	17,34
+4	10,340	253,000	3,750	240,40
+6	8,730	195,000	1,530	210,70
+8	3,650	76,300	1,150	79,50
+10	1,950	12,500	738	23,50
+15	1,235	9,350	650	12,60

[a] Groups of five to six mice first injected iv with 4×10^8 SRBCs and then given a secondary injection later with same dose of erythrocytes on indicated day before assay for splenic PFCs.
[b] Mice injected i.p. with 0.5 ml AF on day −1 and 0 before secondary immunization with SRBC.
[c] Indirect 7S IgG PFCs detected by facilitation procedure with antiglobulin serum.

pression of the IgM as compared to the IgG response. These results point to the need for examining several parameters of the immune response, even to the same antigen, in order to determine mechanisms. The lack of a significant depression of IgG PFCs after secondary immunization suggests that the mastocytoma immunosuppressive factor(s) preferentially affects those immunocytes forming macromolecular hemolytic antibodies, i.e., 19S IgM hemolysis. On the other hand those immunocytes which develop later in the primary immune response and in larger numbers during the secondary response to SRBCs and secrete 7S hemolysins are essentially unaffected. These cells, presumably B-lymphocytes, require T-cells for antibody formation.

Results of this study appear to be at variance with earlier studies from this laboratory in which it was shown that "educated" T cells, when added to immunosuppressed spleen cell suspensions from mastocytoma bearing animals, restored immune responsiveness. It seemed likely from that study that a major immunologic defect caused by mastocytomas, at least in terms of responsiveness to SRBC, was related to helper T cell functions (9). If this were so, then mastocytoma treated mice should show a preferential impairment of the IgG rather than the IgM PFC response, since the "switch" to IgG antibody formation is thought to depend on T cell production. However, differences between *in vivo* vs. *in vitro* responses may explain this apparent inconsistency. Factors which influence the immune response may not be important or may be l during *in vitro* conditions. Also, helpe activity for anti-SRBC responses may pressed through different mechanisms mary vs. secondary responses.

The results of the present studies sug that "conversion" of IgM to IgG an responsiveness in vivo is less affecte formation of IgM antibody per se als trasts with the widely accepted view th antibody formation depends upon a ing IgM antibody response (10). How is not yet clear whether separate cla precursor cells exist for these two cell lations. Regardless of the mechani volved, it seems apparent from the re this study that mastocytoma-bearing m se as well as mice treated with AF tumor-bearing animals show a prefe depression of the IgM antibody respc compared to the IgG response.

Summary. Immunologic impairme curs in mice bearing mastocytoma tun injected with cell free extracts and/or fluids from the tumor bearing animals. present study it was found that the 19 antibody response to sheep red blood assessed at the individual hemolytic forming cell level was preferentially a Whereas there were 60–80% fewer IgM in mice given a primary injection with erythrocytes, there was only a slight e appearance of indirect 7S IgG plaqu ing cells. Furthermore, mice prime

sheep red blood cells and then implanted with mastocytoma cells showed a marked impairment of the secondary IgM response but no depression of the IgG response. Injection of cell free ascites fluid derived from mastocytoma-bearing mice into normal recipient mice before primary or secondary immunization with sheep red cells resulted in marked depression only of the IgM response. These results indicate that mastocytoma affects preferentially in immunocytes producing IgM hemolytic antibody and not those involved in formation of 7S antibody.

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Bile Acids in Bile of Germfree and Conventional Dogs[1] (40059)

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Germfree dogs have been reared for a number of years, mostly to be used for the study of various surgical problems (1). However, they are also of interest to the gnotobiologist because they are mammals not subject to the usual cecal enlargement found in the germfree rodent. The gastrointestinal function of the dog may possibly be more similar to man's than that of the rodent, and with this in mind, a collaborative study of the gastrointestinal function of the germfree dog was begun.[2] Gallbladder bile from germfree and conventional dogs was available for a preliminary study of the dog as a model for the investigation of sterol metabolism. This is the first report on the biliary bile acids of the germfree dog.

In addition to being the main catabolic end product of endogenously and exogenously derived cholesterol,[3] the actual presence of bile acids in the gut affects a number of intestinal parameters. They influence the absorption of lipids (2), but may also affect Ca and Mg absorption (3) since, in the gut a substantial part of these metals may be bound to fatty acids (4). They appear to affect the renewal rate of the intestinal mucosa (5, 6) and aspects of intestinal metabolism (7, 8), including transport related ATP-ases (9).

The absence of an intestinal microflora leads to extensive qualitative and quantitative changes in bile acids of rats and mice (10, 11). In conventional rabbits the major biliary bile acid is deoxycholic acid, which is absent in the bile of the germfree rabbit (12). Preliminary data comparing germfree and conventional gerbils indicate chenodeoxycholic acid as a major bile acid in germfree, but not conventional gerbils maintained at the Lobund Laboratory.[4] In bile of germfree piglets hyocholic acid predominates, while in conventional piglets hyocholic and hyodeoxycholic occur in comparable amounts (13). The data in this report show much less extensive differences between germfree and conventional dog bile, with cholic acid being the dominant bile acid in both cases.

Materials and methods. Pure-bred germfree and conventional Beagle dogs of mixed sexes and 5–6 months old were obtained from Louisiana State University Medical Center, Department of Surgery in New Orleans, Louisiana. After caesarian birth the germfree puppies had been housed in isolators described by Heneghan, Longoria and Cohn (14) and fed autoclaved Borden Esbilac (The Borden Co., New York, NY) with 1 ml filter sterilized Vi-Syneral Multiple Vitamins (B vitamins plus vitamins A, D, E, and C) (U.S. Vitamin and Pharmaceutical Corp., New York, NY) per liter of milk diet. A filter-sterilized 2.5% methionine solution had been added to the milk so each puppy received approximately 30 mg of methionine per day until they were weaned to autoclaved Purina

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[2] This project involved Drs. J. B. Heneghan, Louisiana State University Medical Center, New Orleans, LA; Helmut A. Gordon, University of Kentucky Medical Center, Lexington, KY; Silvio Baez, Albert Einstein College of Midicine, New York, N.Y.; Esther Kokas, University of North Carolina, Chapel Hill, N.C.; and Bernard S. Wostmann, University of Notre Dame, Notre Dame, IN. Preliminary results were presented at the 11th Annual Meeting of the Association for Gnotobiotics, Guelph, Ontario, Canada, June 9–13, 1974.

[3] The following systematic names are given to bile acids and sterols referred to by trivial names: cholesterol—cholest-5-en-3β-ol; cholic acid—3α,7α,12α-Trihydroxy-5β-cholanic acid; α-muricholic acid—3α,6β,7α-Trihydroxy-5β-cholanic acid; β-muricholic acid—3α,6β,-7β-Trihydroxy-5β-cholanic acid; Chenodeoxycholic acid—3α,7α-Dihydroxy-5β-cholanic acid; Deoxycholanic acid—3α,12α-Dihydroxy-5β-cholanic acid; Hyodeoxycholanic acid—3α,6α-Dihydroxy-5β-cholanic acid; Lithocholanic acid—3α,Hydroxycholanic acid; 7-ketolithocholic acid—3α-Hydroxy-7-ketocholanic acid; 7-ketodeoxycholanic acid—3α,12α-Dihydroxy-7-keto*cholanic* acid.

[4] Wostmann, B. S. Unpublished data, 1975.

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:how (Ralston Purina Co., St. Louis, upplemented with 1.25 ml Vi-Syneral ins per liter of drinking water. Conven- control animals had been housed in rd stainless steel cages, two to a cage open animal facility, and were fed ived Purina Dog Chow and water *ad*

ples of gallbladder bile were obtained psy, and diluted 1:20 with 50% (v/v) l for storage. Bile acids were prepared methodology (15) which is summa- iere. For analysis, ethanol was added liquot of diluted bile sample to a final l concentration of approximately 75% and NaOH was added to 0.50 *N*. Car- e cholic acid-[^{14}C] was added to deter- ecovery after sample preparation (see er). The mixture was refluxed 1 hr team, cooled, and the neutral sterols ed by four extractions with hexane. ater phase, containing the bile acids, ade more basic (0.8 *N*), and the mixture ived to hydrolyze conjugated bile After cooling, the mixture was acidified ICl and bile acids were extracted once hloroform:methanol (2:1) and thrice hloroform. Bile acids were methylated ght by dissolving in 10 ml of 5% ace- ride in methanol.

material was streaked onto 20 × 20 cm yer chromatographic (TLC) plates (250 ick), and developed once in chloro- enzene (1:1) to remove fatty acids. The was then developed in isooctane:iso- ol:HAc (100:60:1) to carry the bile way from the origin, the latter contain- ost of the pigmented material. After of bile acids from the gel with acetone, ere streaked on a second TLC plate developed in chloroform:acetone: ol (70:25:5); this separates the bile ixture into three fractions suitable for uid chromatography (GLC) analysis: zone of trihydroxy bile acids, a middle ontaining hyodeoxycholic acid (when t) and the upper zone carrying all di- y and keto-BAs (15).

n internal standard for GLC quanti- 5α-cholestane was added to the eluted id fractions. An aliquot representing rth of the sample was used to deter- holic acid-[^{14}C] recovery to correct for losses during the foregoing analytical procedure. One milligram methyl cholate carrier was added and the mixture incorporated in 10 ml 2,5-diphenyloxazole (PPO) (5 g/l) in toluene, with 0.5 ml of methanol added to assure complete solution of all material. Radioactivity was determined with a Beckman Instruments LS-250 Liquid Scintillation System using the automatic quench correction (AQC).

The remaining three-fourths of the sample was dried and the bile acids converted to trimethylsilyl (TMS) ethers for flame ionization GLC on 1% SE-30 and on 3% QF-1, both supported on 100/200 mesh Gas Chrom Q (Applied Science Laboratories, Inc., State College, PA), for quantitation and identification (15). Quantitation was done by integration of GLC peaks with an Autolab System IV Computing Integrator (Autolab Division of Spectra-Physics, Santa Clara, CA).

Quantitative corrections were made based on the recovery of the cholic acid-[^{14}C]. Recoveries were always better than 85%.

Results. With the limited number of dogs available no obviously significant difference between total bile acids in bile of germfree and conventional dogs could be found (Table I). The bile acids of all dog bile samples, from both germfree and conventional animals ranged from 23 to 44 mg/ml bile, with the germfree dogs tending to show higher values. In both germfree and conventional dogs, cholic acid was the dominating component, with approximately 4% chenodeoxycholic being present. In the conventional animals 10–20% deoxycholic acid was present, accounting for the decrease in cholic acid. Only traces of keto-bile acids were found in the bile of either group, usually with a GLC

TABLE I. BILE ACIDS IN GALLBLADDER BILE OF ADULT GERMFREE (GF) AND CONVENTIONAL (CV) BEAGLE DOGS.

	GF (8)[a] %	CV (4) %
Cholic acid	94.9 ± 0.7[b]	81.6 ± 2.2
Chenodeoxycholic acid	4.6 ± 0.6	3.7 ± 0.6
Deoxycholic acid	—	12.1 ± 3.1
Ketones	trace	trace
Total bile acids mg/ml	38.8 ± 2.6	28.5 ± 2.9

[a] Number of animals in parenthesis.
[b] Mean ± SE.

retention time that suggested 12-keto-3α cholanic acid.

Discussion. Gans and Butz (18) found gallbladder bile acids in conventional dogs to consist largely of taurocholate and taurodeoxycholate. Earlier, Gans (19) had reported 30–40% taurodeoxycholate determined by a chemical method. The present data on conventional dog material are more in line with the findings of Quarfordt and coworkers (20). Using conventionally reared mongrel dogs, they found the biliary bile acids to be cholic (69%), chenodeoxycholic (9%) and deoxycholic (21%) acids with total bile acid concentration of 20.2 mg/ml bile. Differences between these and the present data may be due to dog breed, diet or different methods of quantitation.

The predominant bile acid of the germfree Beagle dog is cholic acid; this, together with the other primary bile acid, chenodeoxycholic acid, represents at least 99.5% of the total gallbladder bile acids. In conventional dogs, deoxycholic acid is presumably formed by bacterial degradation of cholic acid in the gut and recirculates via the liver without being completely rehydroxylated. Chenodeoxycholic acid occurs in approximately the same quantity as found in the germfree animal. Cholic acid, deoxy- and chenodeoxycholic acids represent 99.3% of the material found in the bile acid fraction of the conventional dog bile.

Biliary bile acid composition reflects, to a large extent, the qualitative composition of bile acids that are functionally active in the small intestine. In the most frequently used animal models, the rat and the mouse, bile acid composition is considerably different from that in man (Table II). Although the bile acids in dog and human bile are not quantitatively comparable, they are much more similar than are those of rodents and man. Human gallbladder bile consists mainly of cholic acid (44%), chenodeoxycholic acid (33%), and deoxycholic acid (19%) representing 96% of the total bile acids with the remainder lithocholic acid (4%) (21). Total bile acid concentration falls in the same range as found for the dog (Table II), but 75% of human biliary acids are conjugated with glycine and only 25% with taurine (23), whereas almost all the bile acids in both dog (18) and rat (24) bile are taurine conjugated. β-Muricholic acid, very much in evidence as a primary bile acid in rat (10) and mouse (11) bile, is present neither in dog nor in human bile.

Moreover, the bile acid patterns of the germfree rat and mouse differ very substantially, both quantitatively and qualitatively, from those of their conventional counterparts (10, 11). In the dog, germfree and conventional animals differ only in the presence of a moderate amount of deoxycholic acid in conventional dog bile. Total amounts of bile acids appear to fall in the same range, although there seems to be a trend towards higher values in the germfree animal reminiscent of the more pronounced differences found in murine rodents (10, 25). Evidently

TABLE II. PERCENTAGE OCCURRENCE OF MAJOR BILE ACIDS IN BILE OF VARIOUS MAMMALIAN SPECIES.

	Dog		Rat (10)		Mouse		Human	
Bile Acid	GF	CV	GF[a]	CV[a]	GF (11.25)	CV[b]	GB (23) feces[c]	CV (21)
Chenodeoxycholic acid	4.6[d]	3.7	0.6	4.2	1.5	—	++	33.4
Cholic acid	94.9	83.6	49.4	74.8	24.5[e]	52.9	++	44.2
Deoxycholic acid	—	12.1	—	1.0	—	3.5	—	18.6
β-muricholic acid[f]	—	—	49.2	15.3	68.0	37.5	—	—
Hyodeoxycholic acid	—	—	—	3.0	—	trace	—	—
Bile acids total (mg/ml)	36.8	28.5	10.12	4.16	not given		?	37.9

[a] Lobund rats of Wistar origin.

[b] Wostmann, B. S. Unpublished data, 1975.

[c] 2 gnotobiotic (GB) children, one GF 3 months then contaminated with a bacterium of the *Klebsiella-aerobacter* group and *Candida albicans* and the other with a spore-former.

[d] Percent of total bile acids.

[e] Includes 1.7% of cholic acid-7-SO_4 derived from cholic acid, according to personal communication from H. *Eyssen,* University of Leuven, Belgium, 1974.

[f] *This fraction includes 5–10%* α-muricholic acid (10).

stinal microflora exerts far less influ-
the bile acid pattern of the dog than
of any of the other species studied

present data indicate similarities that
the possible usefulness of the dog as
l for the study of the microbial factor
acid and cholesterol metabolism as it
o cardiovascular disease in man. The
tedly small differences, both quali-
nd quantitative, between bile acids of
e and conventional dogs suggest that
ral, germfree dogs may be used ad-
ously in studies of intestinal function
tabolism that require absence or con-
he intestinal microflora. The similar-
ile acid composition would warrant
umption that bile acid-dependent
n in the small intestine of gnotobiotic
uld be comparable to that of conven-
nimals.

nary. The amount and composition of
lder bile acids of eight germfree and
nventional pure-bred Beagle dogs
termined. Unlike in other mamma-
cies, no major differences in bile acid
ition were found between germfree
nventional dogs. Germfree dog bile
ed on the average 94.9% cholic acid
% chenodeoxycholic acid. Conven-
log bile contained 83.6% cholic acid,
enodeoxycholic acid and in addition,
eoxycholic acid. Bile from both germ-
l conventional dogs contained traces
-bile acids. The average amounts of
e acids in mg/ml bile were 36.8 ± 2.6
ee) and 28.5 ± 2.9 (conventional).
for differences in conjugation, dog
nan biliary bile acid patterns are qual-
comparable, and differ from those in
l mice in that muricholic and related
ls are found only in the latter group.

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The Distribution of Blood Flow Along the Small Intestine of The Dog (40060)

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The presence of a blood-flow gradient along the small intestine is disputed. Geber (1), using electromagnetic flowmeters in dogs, and Steiner and Mueller (2) using the indicator fractionation technique in rats demonstrated that blood flow per unit weight was highest in the duodenum and then decreased progressively along the jejunum, ileum, and colon. On the other hand, Grim and Lindseth (3) using direct collection of venous outflow isolated loops of intestine in the dog, and Delaney and Custer (4) using the indicator fractionation technique in dogs did not observe a blood flow gradient. The negative view has also been supported by a more recent study of Goodhead (5) using the indicator fractionation technique in the dog.

This paper presents evidence that a blood flow gradient does exist in the small intestine of the dog when it is studied under basal conditions of anesthesia.

Materials and methods. Eighteen mongrel dogs weighting 15–30 kg were given water but no food for 24 hr in the preparation period. General anesthesia was then induced and maintained with intravenous pentobarbitone sodium (30 mg/kg). Blood pressure and heart rate were monitored from a femoral artery catheter and body temperature was kept at 37.5°. Care was taken to perform all blood flow measurements when these functions were stable.

Intestinal blood flow was measured by the indicator–fractionation technique with ^{42}K (15 μCi/kg) as the indicator. The ^{42}K was injected into the inferior vena cava and tissue ^{42}K content 100 sec after the injection was used for all blood flow calculations (5–8). The cardiac output was measured by the conventional dye dilution technique (9) immediately prior to the injection of the ^{42}K. Cardiac arrest was produced by a rapid injection of saturated magnesium sulphate into the inferior vena cava and this enabled removal of 3–5 cm sleeves of intestine for ^{42}K assay from the third part of the duodenum, the jejunum (10 cm distal to the ligament of Trietz), and the distal ileum (30 cm proximal to the ileo-caecal valve). Additional 3–5 cm sleeves of intestine were also taken from the jejuno-ileum in eight of these dogs. The mesentery and excess fat were removed, the intestine tissue weighed and then ^{42}K was measured in a Baird atomic γ-well counter.

All animals were studied under anesthesia with no attempt to vary the intestinal blood flow. Seven dogs were studied without prior laparotomy but eleven dogs had a midline laparotomy to enable identification of intestinal regions before the blood flow measurements were made. In these dogs, the midline incision was held closed by metal clips to permit rapid removal of intestinal tissue following cardiac arrest.

Results. Table I shows the blood flow per 100 g tissue in the intestine of the 18 dogs studied. On a two-way analysis of variance there was a significant difference of blood flow in the duodenal, jejunal and distal ileal sites ($P < 0.01$), and on Scheffé multiple comparison tests the mean blood flow in the distal ileum was significantly lower than in the duodenal site ($P < 0.05$) or jejunal site ($P < 0.05$). There was no significant difference of blood flow between the duodenal and jejunal sites. In the 8 studies in which jejuno-ileal blood flow was also measured, the mean blood flow in the distal ileum was significantly lower than in the jejuno-ileal site ($P < 0.01$). Blood flow in the jejuno-ileal site was lower than in either the duodenal or jejunal sites but this difference was not significant ($P > 0.05$).

In the 18 studies the mean intestinal blood flow to all sites was significantly higher ($P < 0.05$) in those with cardiac output > 10 ml/min per 100 g. This is reflected by a generally higher blood flow to specific intestinal regions in the seven dogs without laparotomy since the cardiac output was somewhat higher in these animals (14.1 ml/min per 100 g, s.d. 3.2 ml) than in the 11 with

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TABLE I. INTESTINAL BLOOD FLOW MEASURED BY THE INDICATOR FRACTIONATION TECHNIQUE IN ANESTHETIZED DOGS (ml/min per 100 g INTESTINAL TISSUE).

Dog No.	Cardiac output (ml/min per 100 g body wt)	Duodenum	Jejunum	Jejuno-ileum	Distal-ileum
		No laparotomy prior ^{42}K injection			
1	11.0	58	59		30
2	9.6	60	59		42
3	14.3	68	72		47
4	13.5	71	70		48
5[a]	18.8	78	80		175
6	16.6	92	92		64
7	15.0	99	93		47
s 1–7 Mean (s.d.)	14.1 (3.2)	75 (16)	75 (14)		65 (49)
; 5[a] excluded:					
lean (s.d.)	13.3 (2.6)	75 (17)	74 (15)		46 (11)
		Laparotomy prior to ^{42}K injection			
8	7.1	36	36	25	21
9	7.6	39	35	33	31
10	9.2	41	43	44	37
11	7.5	46	48		32
12	6.5	47	45	44	35
13	8.0	53	61	57	34
14	6.6	56	50	45	38
15	11.8	56	73		38
16	10.7	64	75	57	42
17	11.8	78	76	71	42
18	15.0	103	110		52
s 8–18 Mean (s.d.)	9.3 (2.7)	56 (20)	59 (23)	47 (14)	37 (8)

[a] Ileal sample heavily bile stained (see text).

arotomy (9.3 ml/min per 100 g, s.d. 2.7

n these studies only one animal (Dog 5) l a large amount of bile in the distal ileum, l this was the only animal in which blood v in the distal ileum was greater than in ıer the duodenal or jejunal sites. If this mal was excluded from analysis, then the erence between blood flow in the distal l site and the duodenal or jejunal sites was n more significant ($P < 0.001$).

Discussion. The experiments show that en intestinal blood flow is measured under al conditions in the dog there is a definite dient of blood flow down the intestine. w is highest in the duodenum and je- um, and diminishes progressively in the ıno-ileum and distal ileum. In the distal ım the mean flow is only 65% of that in duodenum or jejunum. This gradient was erved whether or not the abdomen had n opened prior to the measurement. In dog the blood flow gradient was reversed, distal ileum having more than twice the od flow (per unit weight) of the duodenum l jejunum. In this study, bile was observed in the lumen of the distal ileum, and since bile salts are absorbed in this region by a highly specific, energy dependent, active transport mechanism (10) it is possible to speculate that bile salts may stimulate blood flow in this region.

In the seven studies in which laparotomy was not performed, the duodenal and jejunal blood flow averaged 75 ml (s.d. 14) per min for 100 g tissue. This is very similar to the figure of 72 ml/min reported by Delaney (11) in dogs and the 68 ml/min noted by Ross (12) in cats. In the 11 basal studies in which laparotomy had been performed, the duodenal and jejunal blood flows averaged 58 ml (s.d. 21) per min for 100 g tissue. This reduction in intestinal blood flow is of the same magnitude as the reduction in gastric blood flow noted by Delaney and Grim (13) as an effect of laparotomy. The blood flow figure of 58 ml/min per 100 g when the abdomen had been opened is very similar to the figure of 60 ml/min per 100 g noted by Grim (14) using a direct bleed-out technique in isolated loops of intestine in the dog.

In previous studies several authors (3–5)

have not observed a blood flow gradient down the intestine when measurements were made under basal conditions. However, these papers are a little misleading, since Goodhead (5) did observe differences but was not able to demonstrate statistical significance because his measurements showed a s.d. of 50%. In the paper by Delaney and Custer (4) the blood flow was measured in only two sites, the duodenum and the rest of the small intestine as a whole; in these sites the blood flow figures of 70 ml and 72 ml/min per 100 g tissue were not significantly different. Since blood flow in the jejunum, jejuno-ileum and distal ileum was not measured separately, the differences which we report might well have been obscured. Finally, the study of Grim and Lindseth (3) measured jejunal and ileal blood flow by direct collection of venous outflow from isolated loops of intestine. The operative manipulations involved in preparing an isolated loop, and in particular the cannulation of blood vessels may have modified blood flow and thus prevented differences from being observed.

Whatever the problems may have been in these earlier studies, the data presented in this current paper clearly support the observation of Geber (1) and Steiner and Mueller (2) that under basal conditions there is a blood flow gradient down the intestine, with blood flow (per unit weight) highest in the duodenum and lowest in the distal ileum.

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ndependent Effect of Bicarbonate on Renal Citrate Metabolism[1] (40061)

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citrate metabolism in rat, dog and ɔears to be a pH dependent reaction ı addition to the role played by pH e data indicating that renal citrate ism is affected by bicarbonate ions from any effect on blood pH. Simp- for example, has demonstrated that ɔtic dogs increasing the bicarbonate ation isohydrically raises the renal learance. Potassium ions also appear ıportant in controlling renal citrate ism since hypokalemia decreases rate clearance (5). By contrast, acute ration of KCl although increasing itrate does not appear to alter renal ontent or excretion (6). Thus, both ıate and potassium seem to regulate ıetabolism but the interrelationships them are unknown. To study this the blood bicarbonate concentration red isohydrically in normokalemic, lly hypokalemic and acutely hyper- rats and renal citrate content and citrate excretion measured. The re- w that bicarbonate, separate from its ı blood pH, alters renal citrate metab- all levels of serum potassium.

ds. Intact male Sprague–Dawley rats g 350–500 g were anesthetized with ınd prepared surgically as previously d (7). Unless otherwise noted animals used intravenously with 2.1 ml saline r and ventilated with a gas mixture ng 30% oxygen and 70% nitrogen. In riments a 60 min control period was l by a 150 min experimental period. re sacrificed by exsanguination from ominal aorta into a heparinized sy- enal cortical tissue was removed and frozen in liquid nitrogen for citrate as previously described (5). Each urine was filtered and analyzed for ıe and citrate.

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Normokalemic experiments. Following the control period, rats were divided into three bicarbonate groups. The unchanged bicarbonate group, was ventilated with a 30% oxygen–70% nitrogen gas mixture at a rate of 120/min and a tidal volume of 2.0 ml while receiving 2.1 ml of saline per hr. The lowered bicarbonate group was hyperventilated with the 30% oxygen–70% nitrogen gas mixture at a ventilatory rate of 140/min and a tidal volume of 2.25 ml. Simultaneously, the venous infusate was changed to 0.2 *N* HCl in saline given at approximately 2.1 ml/hr. The elevated bicarbonate group, was ventilated with a 10% carbon dioxide, 30% oxygen, 60% nitrogen gas mixture at a rate of 120/min and a tidal volume of 2.0 ml. Simultaneously, the venous infusate was changed to a 0.35 *M* sodium bicarbonate solution given at approximately 2.1 ml/hr. Mean total sodium administered to the normal, lowered and elevated bicarbonate groups in the experimental period was 1.28, 1.28 and 1.46 mEq respectively. In all groups 0.2 ml blood samples were taken at 15 min intervals for the first hour of the experimental period to ascertain constancy of blood pH. This was not done in all animals once the procedure had been standardized. This same protocol was employed in hypokalemic and hyperkalemic animals. Other normokalemic rats were prepared in the same manner but acute respiratory acidosis was induced by ventilation with 10% carbon dioxide, 30% oxygen and 60% nitrogen mixture.

Chronic hypokalemia protocol. Rats were placed on a low potassium, low sodium diet (obtained from Nutritional Biochemical Corporation, Cleveland, OH). Animals were allowed water containing 75 mEq/liter of sodium bicarbonate *ad libitum* during the entire study. Rats were maintained on this diet for 15 days. On each of days 8 through 12 rats were given 2 mg of furosemide intraperitoneally.

Acute hyperkalemia protocol. Rats were tube fed twice on the day prior to the proce-

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dure and once on the morning of the experiment. Each feeding consisted of 2 ml of 1 mg/ml triamterene solution and 10 ml of a 5 mEq/liter potassium chloride solution in 5% dextrose and water. In addition, a solution containing 5 mEq/liter potassium chloride was given intravenously during the experimental period at 6.0 ml/hr.

Analytic methods. Arterial pH and pCO_2 were measured in a Radiometer BMS3 MK-2 blood microsystem and bicarbonate calculated from a nomogram. Renal cortical citrate content, blood citrate, and urinary citrate were measured enzymatically employing citrate lyase (8). Electrolytes and urinary creatinines were determined by flame photometry and standard autoanalyzer techniques.

Results. Blood acid-base and potassium values (Table I). The mean serum potassium concentration of the 24 normokalemic rats shown in Table I was 4.3 ± 0.4 mEq/L in the control period. Control blood pH values did not differ among the three isohydric normokalemic bicarbonate groups and the pH did not significantly change in the experimental period ($P > 0.4$). The blood pH of the hypokalemic and hyperkalemic rats also did not significantly differ among the three isohydric groups ($P > 0.4$) and there were no significant differences between the control and experimental period pH values in any group ($P > 0.5$). The change in bicarbonate concentration in the lowered and elevated bicarbonate groups in each of the three potassium states was highly significant ($P < .001$) at the same time bicarbonate concentration did not significantly alter in the unchanged groups ($P > 0.1$). Serum potassium concentration in the 26 hypokalemic and 19 hyperkalemic rats during the control period was 1.8 ± 0.4 and 7.0 ± 0.4 mEq/L respectively.

Effect of bicarbonate concentration on blood citrate concentration. Isohydric changes in blood bicarbonate did not affect the blood citrate concentration. As shown in Table I although blood citrate rose in the experimental period in all the normokalemic and hypokalemic bicarbonate groups there were no differences in blood citrate among these groups in the experimental period ($P > 0.2$). In hyperkalemic rats blood citrate did not rise in the experimental period ($P > 0.5$) and there were no differences between groups at the end of the experimental period ($P > 0.4$).

Effect of respiratory acidosis on renal citrate content. Acute respiratory acidosis was induced in six normokalemic rats. Blood pH fell from 7.42 ± .02 to 7.06 ± .02 with the acute elevation of pCO_2 from 35 ± 2 to 70 ± 2 mmHg and blood bicarbonate remained unchanged at 22.5 ± 2.2 mEq/L in the control period and 20.6 ± 2.6 mEq/L during the acidotic period. Renal cortical citrate content was 0.132 ± 0.009 μmole/g in the acidotic rats compared to 0.232 ± 0.035 μmoles/g in the normokalemic rats with unchanged bicarbonate.

Effect of bicarbonate concentration on renal citrate content. Renal citrate content in normokalemic rats with unchanged bicarbonate

TABLE I. BLOOD VALUES IN NORMOKALEMIC, HYPOKALEMIC AND HYPERKALEMIC RATS[a].

	Bicarbonate mEq/L		Arterial pH		Citrate μmoles/ml	
Group (n)[b]	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.
Normokalemic						
U (7)	25.0 ± 1.4	24.3 ± 1.6	7.35 ± 0.04	7.34 ± 0.05	0.076 ± 0.020	0.109 ± 0.038
L (10)	21.0 ± 2.4	10.9 ± 2.8[c]	7.36 ± 0.05	7.33 ± 0.05	0.069 ± 0.026	0.116 ± 0.039
E (7)	21.5 ± 1.5	44.3 ± 5.6[c]	7.39 ± 0.02	7.40 ± 0.03	0.081 ± 0.029	0.132 ± 0.060
Hypokalemic						
U (9)	31.1 ± 3.3	27.0 ± 2.9	7.53 ± 0.09	7.52 ± 0.09	0.104 ± 0.045	0.134 ± 0.057
L (11)	37.9 ± 3.5	21.7 ± 4.1[c]	7.51 ± 0.07	7.52 ± 0.06	0.089 ± 0.028	0.144 ± 0.060
E (7)	34.2 ± 1.3	59.8 ± 3.1[c]	7.51 ± 0.04	7.46 ± 0.06	0.091 ± 0.036	0.120 ± 0.082
Hyperkalemic						
U (6)	21.4 ± 2.0	19.9 ± 2.4	7.27 ± 0.04	7.27 ± 0.05	0.057 ± 0.028	0.060 ± 0.022
L (7)	23.5 ± 0.8	13.4 ± 1.1[c]	7.28 ± 0.05	7.27 ± 0.05	0.076 ± 0.034	0.075 ± 0.037
E (6)	20.7 ± 2.7	43.9 ± 5.4[c]	7.29 ± 0.05	7.31 ± 0.03	0.049 ± 0.028	0.069 ± 0.024

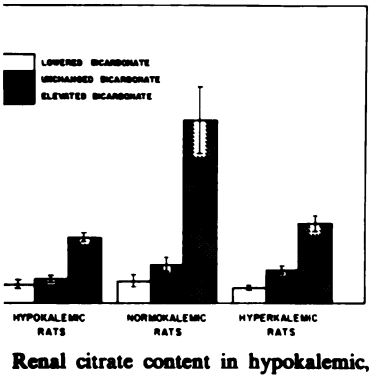
[a] All values are expressed as the mean ± SEM. Cont. is control period, exp. the experimental period.

[b] Numbers in parentheses refer to number of animals in each group. U is unchanged, L, lowered, and E, elevated bicarbonate groups.

[c] Significantly different from control ($P < 0.01$).

32 ± 0.035 μmoles/g compared to
f 0.145 ± 0.016 ($P < .01$) and 0.199
($P > 0.2$) in hypokalemic and hyper-
unchanged groups respectively. As
n Fig. 1 at each level of plasma po-
raising the bicarbonate isohydrically
d renal citrate content significantly
)01). Lowering the bicarbonate con-
)n, however, significantly decreased
trate content only in the normoka-
$^2 < 0.02$) and acutely hyperkalemic
< 0.005).

of bicarbonate concentration on urine ccretion. While increased bicarbonate
rine citrate excretion at each level of
m (Fig. 2) statistically significant in-
were noted only when the lowered
ated bicarbonate groups at each po-
level were compared ($P < .01$). Fig-
)o shows that as was found with the
trate content the greatest absolute
in urinary citrate excretion occurred
evated bicarbonate groups. Respira-
losis without change in blood bicar-
ad no effect ($P > 0.4$) on urine citrate
1 with 1.48 ± 0.47 μmoles/mg Crt
d to 1.62 ± 0.6 μmoles/mg Crt in
ılemic unchanged bicarbonate rats.
:sion. The present experiments clearly
it bicarbonate's effect on renal citrate
ism, separate from pH, is operative at
na potassium levels. These results
the work of Simpson who earlier
that isohydric increases in bicarbon-
entration raised the urinary citrate



Renal citrate content in hypokalemic, nor-
, and hyperkalemic rats. The white bars rep-
ered bicarbonate, the black bars unchanged
e, and the hatched bars elevated bicarbonate
ats at each potassium level. Each bar indicates
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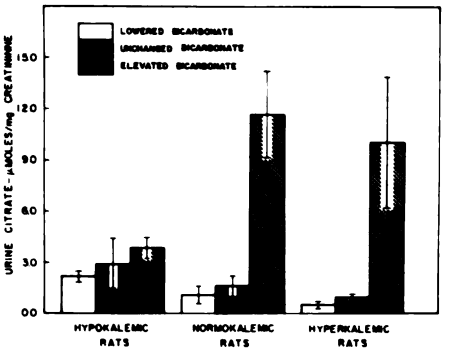

FIG. 2. Urine citrate excretion in hypokalemic, normokalemic, and hyperkalemic rats. The white bars represent lowered bicarbonate, the black bars unchanged bicarbonate, and the hatched bars elevated bicarbonate groups of rats at each potassium level. Each bar indicates the mean ± SEM.

clearance of alkalotic dogs (4). However, Simpson was unable to demonstrate a similar effect in acidotic dogs. Our data suggest that his inability to demonstrate any effect of changes in bicarbonate concentrations in acidosis is due to the already markedly decreased urinary citrate clearance in acidosis and thus inability to separate the subgroups. Thus, in our experiments the reduction in urinary citrate excretion induced by lowering bicarbonate in normokalemic rats was not statistically significant while at the same time renal cortical citrate content did decrease significantly in these same animals. Similar results were seen in the hyperkalemic rats where lowering the bicarbonate concentration significantly reduced renal citrate content but not urinary citrate excretion. Only in hypokalemia was a reduction in bicarbonate not associated with a significant decrease in renal citrate content. This may be a consequence of the already significantly reduced cortical citrate content induced by hypokalemia.

The renal citrate content of normokalemic rats with lowered bicarbonate is almost identical to that found in rats with pure respiratory acidosis and a constant bicarbonate concentration. Thus, renal citrate content fell to 0.132 μmoles/g, a value not significantly different from the 0.135 μmoles/g measured in the lowered bicarbonate normokalemic rats or the 0.145 μmoles/g values seen in the unchanged hypokalemic rats. Thus, acidosis without a change in bicarbonate reduces

renal cortical citrate content to the same extent as does hypokalemia or an isohydric reduction in bicarbonate concentration.

The experiments also show that urinary citrate excretion increased when the bicarbonate concentration was elevated. This effect, which was demonstrable in each of the three potassium states, could be due to an increased filtered load of citrate, increased renal citrate production, or decreased renal tubular citrate reabsorption and utilization. Since bicarbonate elevation did not alter blood citrate levels, and as urinary citrate excretions were calculated per unit of creatinine excreted thereby correcting for any changes in GFR, the increased urinary citrate cannot be due to an increase in filtered load. It is also unlikely that increased renal citrate production is responsible for the increased urinary citrate. Citrate is primarily produced in intestine and bone (9) and is utilized rather than produced by the kidney. Indeed, in normal acid base and potassium states renal citrate utilization contributes approximately 10% of renal energy needs (10). Also, a variety of experiments have been unable to demonstrate citrate secretion by the mammalian tubule (2). It is probable, therefore, that the increase in urinary citrate is due to decreased renal tubular reabsorption and utilization. Previous clearance studies performed in alkalosis support this conclusion (11). In addition, it has been shown that although incubation of renal cortical slices at varying bicarbonate concentrations does not alter citrate decarboxylation if medium pH is held constant (12), citrate decarboxylation by mitochondria does vary inversely with the bicarbonate concentraiton. From the work of Simpson (13) it has been felt that bicarbonate directly reduces mitochondrial citrate utilization by decreasing the transport of citrate into the mitochondrion. However, the recent work of Robinson *et al.* (14) demonstrated that the decrease in mitochondrial citrate utilization was due rather to bicarbonate increasing citrate efflux from the mitochondria. At high bicarbonate concentration, therefore, less citrate would be metabolized and available for excretion. It seems that the intact animals behave in a fashion similar to mitochondria.

In conclusion, the present experiments add to the growing body of data which show that bicarbonate ions, separate from their effect on pH, alter and regulate metabolic activity (15, 16). This may help explain the greater renal ammoniagenesis found in metabolic acidosis when compared to an identical degree of respiratory acidosis (17). The results thus emphasize the need to control both the pH and the bicarbonate concentration in biological experiments.

Summary. Isohydric alteration of the blood bicarbonate concentration in normokalemic, chronically hypokalemic, and acutely hyperkalemic rats did not affect blood citrate concentration. However, both renal citrate concent and urinary citrate excretion increased in the elevated bicarbonate groups at all potassium levels. Thus, bicarbonate, independent of its effect on pH, affects renal citrate metabolism regardless of the serum potassium level.

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True and False Prophylaxis in Experimental Trypanosomiasis (40062)

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; generally accepted that, during the of prophylaxis with chemoprophylac- nts, the host remains protected against mediate infection, as well as against infections. However, it has been de- l how *Trypanosoma congolense* "broke h the protective cover" of prophylactic ithridium compounds, inducing an in- ent type of infection in the protected ; characteristically, these "break- h" strains, when transferred to un- l animals, lead to a mortal infection as / as the original trypanosome strain (1). diamidine (98/202) of 2-phenyl-thion- ne (2), in subcutaneous doses of 100 protected mice inoculated 20 days /ith multiple lethal doses of *Trypano- enezuelense*; when the reactivity of the ed mice to reinoculation was tested 1 onths later, they developed an inter- t parasitaemia beginning the next 3–4 followed by death (3). The consider- elay in the development of the parasi- and its intermittent form we attribute sidual prophylactic action of the drug, ie corollary that the inoculum injected an assumed period of protection may p much later into a lethal infection. ypothesis that the inoculation may l latent infection in the presence of the /lactic drug without manifesting a de- e parasitaemia, and that the chemo- eutic prophylaxis is not an all-or-none nenon, has been investigated.

erials and methods. T. venezuelense, (*T.* , isolated by Dr. Raul Hernández from of equine "derrengadera" in 1969, was ined since through mouse passages in oratory and used for various chemo- eutic studies. Its virulence for mice and as repeatedly titrated: inocula above rasites killed 95% of the injected ani- l a time interval (7–16 days) varying ing to the heaviness of the inoculum. andard ip inoculum of 5×10^5 para- s used in the present study, induced detectable parasitaemia within 24 hr in 63 ± 3%, within 48 h in 90 ± 2%, within 72 hr in 94 ± 1.6% of mice. The progressive parasitaemia terminated in death 3–6 days after its appearance. The rats were as sensitive as mice to the standard inoculum containing 5×10^5 trypanosomes: parasitaemia appeared in 96 ± 1% of the animals within 24–72 hr, increasing progressively until death which occurred 1–7 (av. 5.4) days later.

In the chemoprophylactic experiments, the sc injection of these drugs to groups of 14–35 mice or rats was followed at various periods by the ip inoculation with 5×10^5 trypanosomes. During the first period (2–3 weeks) after inoculation, daily observations were made, if the animals showed no parasitaemia during this interval, the observations were reduced to alternate days.

The infectivity of a selected number of mice and rats was tested by transferrring blood, and spleen and liver macerates suspended in saline, to groups of 4–5 mice. Occasionally, after a certain period of clearance the animals were reinfected with the standard inoculum and further observed for the development of parasitaemia.

Results. Continuous observation of the infection in 95 mice pretreated with 98/202 (five experiments) revealed that the parasitefree period is drug concentration dependent, since it varied as a function of the dosage if injected at the same time before inoculation, and, when the same dose was given, it decreased with the interval from the pretreatment. Thus, groups of mice inoculated 20 days after pretreatment with 100 mg/kg 98/202, remained parasitefree for 106–121 days, for 26–41 days with 75 mg/kg, and for 10–13 days with 25 mg/kg; inoculation 55 days after pretreatment demonstrated that 75 mg/kg and 25 mg/kg of 98/202 did not retard the appearance of the parasites, although the treatment often delayed their multiplication and the time of death (Table I). Four mice inoculated 8 days after pretreatment with 12

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mg/kg 98/202 remained parasitefree for 8–14 days and died 17–24 days after inoculation; with the same pretreatment, the inoculation after 25 days did not alter significantly the first appearance of the parasitaemia, yet it depressed its progression rate and in 2/4 animals delayed by 10 days the time of death compared to the controls (which died in 7–8 days). The latent infection has been confirmed in a number of experiments by the infectivity of the organs (spleen, liver, blood) of apparently parasitefree animals. The virulence of the parasites transferred from the intermittent infection following the parasitefree period was not modified in comparison to the strain used for inoculation.

Similar results, even though the clearing period remained shorter, were obtained with pentamidine in four experiments including 108 mice. Doses as high as 75 mg/kg injected 6 days before the inoculation of the trypanosomes maintained the majority of the animals parasitefree for 13–26 days, after which the infection ran its fatal course. In 10 mice inoculated 7 days after pretreatment with 40 mg/kg pentamidine, the appearance of parasitaemia was delayed by av. 4 days (min. 0; max. 10) compared to the not pretreated controls. The progression of the parasitaemia until death was slowed down also: the controls died in 5–6 days; the pretreated ones, in av. 13.5 days (min. 13; max. 20). Inoculation 10 days after pretreatment with 40 mg/kg pentamidine, delayed the appearance of the parasites in 1/10 mice only; 6/10 died with a delay of 9–11 days. Table II illustrates an experiment with pentamidine. These phenomena became magnified by using rats for the prophylactic treatment with pentamidine: inoculation 6 days after treatment with 20 mg/kg gave the impression of prophylaxis for 37.6 days, which by inoculation 11 days after pretreatment was reduced to 26.2 days (Table III). In one rat of the former group (No. 170) the intermittent infection was detected only in a single observation made 49 days after inoculation; this rat reinoculated 26 days later, developed a progressive infection after 3 weeks, which counted from the day of pretreatment, represents a protection of 3 months duration.

However, pentamidine and 98/202 as well as other diamidines possess high clearing action without curative effect in the *T. venezuelense* infection of mice and these observations could be attributed to the specific nature of the drugs, rather than to the manifestation of a general phenomenon. In the chemotherapeutic experiments, by sc injection of the drugs 48 hr after inoculation, the clearing dose (ED_{50}) was 1.25 mg/kg for 98/202, 1 mg/kg for pentamidine, and 0.7 mg/kg for suramin. The diamidines, as reported for the antimonials (4), induced no cure by single administration, which was obtained with suramin, 15 mg/kg being its CD_{50} (unpublished). Therefore, the latter drug with definite curative action against this infection, has been investigated for its prophylactic prop-

TABLE I. FALSE PROPHYLAXIS WITH 2-PHENYL-THIONAPHTENE DIAMIDINE (98/202) IN THE *Trypanosoma venezuelense* INFECTION OF MICE (SAME DOSE, DIFFERENT INTERVALS; EXP. No. 8).

98/202 (mg/kg, sc) Day 1	Mouse No.	Inoculation of *T. venezuelense* on day	First appearance of parasitaemia (days after inoculation)	Died
75	199	9	38	46
75	201	9	24	45
(Controls) 0	213–224	9	1;2	3;4
75	203	23	25	41
75	204	23	22	61
(Controls) 0	251–252	23	1;2	4;4
75	211	55	2	24
75	213	55	1	2
75	214	55	2	7
75	215	55	1	3
(Controls) 0	290–291	55	1;2	4;5

TABLE II. FALSE PROPHYLAXIS WITH PENTAMIDINE IN THE *Trypanosoma venezuelense* INFECTION OF MICE.

Pentamidine mg/kg subcut. Day 1	Inoculation *T. venezuelense* Day	Trypanosomes appeared		No. died inoculation [av. (min.–max.)]
		In No. of mice	Days after [av. (min.–max.)]	
37.5	3	14/14	15 (6–26)	26 (16–35)
37.5	6	8/8	15 (7–19)	22 (15–28)
75.0	6	4/7*	21 (19–26)	32 (24–40)
Controls		5/5	1.3 (1–2)	5.4 (5–7)

* *The ultimate fate of 3/7 mice negative on day 40 was not determined.*

rties by various pretreatment schedules in nice (Table IV). It is obvious that, according o the drug concentration in the host at the eriod of the inoculation, the prophylactic ction may remain temporary, with variable, often very long delay in the development of he infection, or else result in the complete destruction of the parasites.

With suitable dosage schedule of suramin pretreatment, a protracted period of false prophylaxis was obtained in rats. Pretreatment with 5 mg/kg 7 days before inoculation, delayed in 4/6 rats the appearance of parasitaemia by av. 21 days (min. 11; max. 25) and

TABLE III. PROPHYLACTIC EFFECT OF PENTAMIDINE AGAINST *Trypanosoma venezuelense* IN RATS.

Exp. No.	No. rats	Pretreatment (mg/kg, sc, of pentamidine)		Inoculation (days)	First appearance of parasite Death of animal ... day after inoculation					
144	2	Controls	—	(5)	2	2				
					7	7				
	3		20	5	19	32	[a]			
					34	44				
	2	Controls	—	(13)	<3	<3				
					8	8				
	3		20	13	23	25	[b]			
					29	30				
145	5	Controls	—	(6)	2	4	2	2	2	
						N.D.*				
	5		20	6	27	28	40	44	49	[c]
					43	40	54	50		
	5	Controls	—	(11)	2	3	2	2	3	
					6	7	7	7	9	
	5		20	11	23	23	24	25	36	
					32	39	50	36	43	
152	3	Controls	—	(3)	1	1	1			
					6	6	7			
	4		10	3	3	17	17	7		
						N.D.*				
	3	Controls	—	(9)	<2	<2	<2			
					3	2	2			
	4		10	9	2	<2	<2	<2		
					3	2	3	3		

[a] One of three rat parasitefree during 70 days observation.
[b] Blood transfer of 1/3 rat 28 days after inoculation negative: animal cured.
[c] Rat 170 (see text): parasites appeared only 1 day; reinfected 75 days after first inoculation became positive in 0 days, i.e. 101 days after pretreatment.
* N.D. Not determined; infected animals used for different experiments.

TABLE IV. TRUE AND FALSE PROPHYLAXIS WITH SURAMIN IN THE *Trypanosoma venezuelense* INFECTION OF MICE.

Suramin (mg/kg sc) Day 1	Inoculation of *T. venezuelense* (day)	Trypanosomes appeared		Prophylactic effect
		In No. mice	Days after inoculation	
Controls (0)		20/20	2	
1.25	10–15	10/10	2–3	None
2.5	7–15	15/16	4–5	None
5.0	7	5/6	13–41	False in 15/26
5.0	10	8/15[a]	24–45	True in 11/26
5.0	15	2/5	10–24	
10.0	7	1/5	60	True in 4/5[b]
20	7	0/5		True (b)

[a] A strain isolated from mouse No. 2495 manifesting parasitaemia 28 days after inoculation is fully sensitive to Suramin.
[b] Transfer of blood and organs induces no infection.

the time of death by av. 33 days (min. 29; max. 44); 2/6 rats remained parasitefree during 44 days observation and were reinoculated: one developed a progressive lethal infection 4 weeks later (102 days after treatment), the other died, without showing parasitaemia. Postponing inoculation to 11 days after 5 mg/kg suramin treatment, in 1/5 rats the infection was not modified; in 3/5, an intermittent infection developed after 23–44 days, leading to death 20–64 days later. In 1/5 rats a progressive infection started 28 days after inoculation, terminating with death 9 days later. Pretreatment with 2.5 mg/kg suramin followed by inoculation 7 days later, only delayed (4–6 days) in 10/14 rats the appareance of parasitaemia with an intermittent infection in 2/10. These 10 rats died in av. 14.5 days (min. 10; max. 21), compared to 9.7 days (min. 7; max. 13) of the control animals. The virulence and the suramin sensitivity of strains recovered after the temporary protection were not modified.

Discussion. The lack of drug sensitivity and virulence variation of the parasites recovered during or after the period of false prophylaxis minimizes the possibility of biological modifications in the trypanosomes and allows an essentially pharmacological interpretation. The prophylactic actions described can be classed in the following categories: (a) true prophylaxis, total protection, without reappearance of the infective organism, and without infectivity of the blood or organs to other animals; (b) false prophylaxis, spontaneous reappearance of the infection at varying periods after inoculation, time of reappearance being a function of the dosage and time of infection, together with infectivity of the blood and/or organs to other animals; (c) with effective prophylactic drugs at certain concentration levels, both effects may appear in a group of animals; thus, one might determine the "True prophylactic $Dose_{50}$". Presumably, the parameters of the prophylactic effect are represented by the antitrypanosomal action of the drug *per se*, by the drug concentration in the body at time of inoculation, and by the half life of the drug.

The intermittent development of the infection after its delayed appearance is no doubt related to the presence in the host of residual *drug, which* though insufficient for total control of the parasitaemia, still has a moderating effect on its rate of development. Probably only when the drug is totally eliminated does the progressive, lethal course of parasitaemia begin. Circumstantial evidence for the supposition that the reappearance of parasitaemia in the prophylactically protected animals is related to the falling drug concentration consists in the correspondence of the period of the reappearance with that of the termination of the protective effect against inoculation (e.g., 7–8 weeks with 75 mg/kg 98/202). At least semantically, the dose and concentration ranges leading to true prophylaxis, which do not allow the survival of the trypanosomes of the heavy inoculum, may be classed as "trypanosomicidal", while those resulting in false prophylaxis are "trypanosomostatic" since they only delay the development of the inoculum without destroying it. This classification does not necessarily represent the immediate mechanism involved, since adjuvant immunological processes and the concentration/time/effect factors are also involved in the final anti-parasitic action. Thus, in prophylaxis, as in the chemotherapeutic process itself, the drug may have a clearing action, with temporary disappearance of the parasitaemia, often accompanied by an intermittent infection, or else a radical suppressive effect, depending on the nature and dosage of the drug. It is justified to ask to what degree these phenomena are related: our observations in chemoprophylaxis (delay, nonpatent period, intermittency) recall phenomena noticed using subcurative doses in chemotherapy. The duration of the therapeutic clearing effect is dose dependent and possibly, some retention of the drug in the organism may be one of its determinants; this is certainly the case with temporary protection obtained by prophylactic treatment. It would be worthwhile to investigate the problem of the therapeutic clearing effect and of the false prophylaxis in terms of the available and stored drug concentration in the host.

The epidemiological significance of the false prophylaxis is obvious, and its possible extent in other infections, particularly under field conditions, should be taken into account.

Summary. Prophylactic treatment of *Trypanosoma venezuelense* infections in mice

ıduce qualitatively different effects. agents such as the diamidine of 2- -thionaphthene (98/202) and pentam- ıith limited curative effect against the ın, in protected mice maintained a long efree period, followed by an intermit- pe of infection terminating in death. erapeutically highly effective suramin, stered before inoculation in sufficient gives a complete "true" protec- ;ainst the infection; however, if the ıncentration in the host is reduced at e of inoculation, either by diminishing or by delaying the time of inoculation, ıtection becomes only apparent. This prophylaxis consists in a nonpatent day) period during which parasites ɔt seen in the blood, followed by the ttent parasitaemia with lethal result. In a similar group of animals, appropriate dosage schedules may produce either effect, the true or the false prophylaxis.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 402–406 (1978)

Oxygen Consumption and Body Fat Content of Young Lean and Obese (OB/OB) Mice[1] (40063)

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It is now well established that genetically obese rodents have an increased ability to convert dietary energy to body energy relative to their lean counterparts (1). Alterations in energy metabolism, as indicated by decreased rates of oxygen consumption, are observable in obese (ob/ob) mice by 3 weeks of age (2–4); however, few studies have focused on younger obese mice. Marked changes in oxygen consumption of obese and lean mice have been reported at the time of weaning. Oxygen consumption per g body weight decreased by 25–50% within the first several days after weaning (3, 4). These studies and others (5–9) indicate that alterations in energy metabolism may appear very early in the life of obese mice.

The present study was designed to monitor oxygen consumption, an indirect indicator of energy expenditure, of lean and obese mice from birth to 112 days of age; major emphasis was focused on developmental changes prior to 21 days of age. Body fat content of lean and obese mice at 7 and 14 days of age was also determined.

Materials and methods. Heterozygote breeding mice (C57BL/6J ob/+)[2] were housed in solid-bottom cages with wood shavings for bedding. They were fed a stock diet[3] *ad libitum*. Ambient temperature was 25 ± 2°. Pregnant dams were removed from the breeding cages and placed in separate similar cages. Litter size was routinely standardized to six pups per litter; however, in a few instances litters with five or seven pups were utilized. Pups were weaned at 21 days of age unless indicated otherwise.

In the first experiment pups were individually identified at 1 day of age. From 1 to 19 days of age daily oxygen consumption measurements and body weights were obtained on individual pups. Litters were also killed at 7 and 14 days of age. Food residue was removed from the stomach and the carcasses were homogenized in an equal weight of water. Carcass fat was extracted with chloroform/methanol (2:1) and quantitated gravimetrically.

Oxygen consumption measurements and body weights of pups on days 18–20 and again on days 22–24 of age were obtained in the second experiment. Only lean mice were used. In one treatment pups remained with the dam; in the other treatments pups were weaned to a high-carbohydrate or a high-fat diet at 21 days of age. The high-fat diet was formulated to approximately simulate mouse milk on a protein-energy basis (10) and contained per 100 g diet: 43.5 g casein, 32.0 g tallow, 5.0 g corn oil, 9.6 g glucose, 5.0 g cellulose, 4.0 g mineral mix (11), 0.4 g vitamin mix (11), 0.2 g choline chloride and 0.3 g methionine. The high-carbohydrate diet was formulated by replacing the tallow with glucose on an equal-energy basis.

In the last experiment oxygen consumption measurements and body weights were recorded weekly from 3 weeks of age to 16 weeks of age. Lean and obese male and female mice were utilized.

The apparatus described by Watts and Gourley (12) was utilized to obtain estimates of oxygen consumption in the mice. Food was available to the mice until the oxygen consumption measurements commenced. Mice were placed in a flask immersed in a water bath maintained at 30°. After 10 min, five consecutive readings of oxygen consumption were obtained during the next 10 min. Oxygen consumption was then calculated at STP and expressed as ml oxygen consumed

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[2] Jackson Laboratory, Bar Harbor, Maine.

[3] Wayne Lab-Blox, Allied Mills, Inc., Chicago, Illinois.

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per g body weight, per g body weight to the 0.75 power (13) and per animal. Relative treatment effects were very similar whether the results were calculated per g body weight or per g body weight to the 0.75 power, thus only results per g body weight are presented.

Results. Body weight and oxygen consumption of pups from 1 to 19 days of age are presented in Fig. 1. The obese (ob/ob) and lean (ob/+ or +/+) pups were identified retrospectively after the obesity became obvious by visual inspection. Differences in body weight were observed as early as the 6th day after birth. Body weights of the obese mice remained higher than that observed for the lean mice during the remainder of the 19-day study. Obese mice consumed less oxygen per g body weight than lean mice by the 5th day after birth. Statistical differences in oxygen consumption per metabolic body size (g body weight to the 0.75 power) between lean and obese mice were also observed by the 5th day after birth; however, the magnitude of this difference was somewhat less than observed per g body weight. In both obese and lean mice the oxygen consumption per g body weight decreased during the second week of life and then remained lower than values observed during the first week of life. When oxygen consumption of the pups was expressed per animal, obese mice tended to consume less oxygen than the lean mice from the 5th day after birth but the differences were significant only on days 5, 12–15 and 18–19. Both obese and lean mice exhibited a five- to sixfold increase in oxygen consumption per animal during the first 19 days after birth.

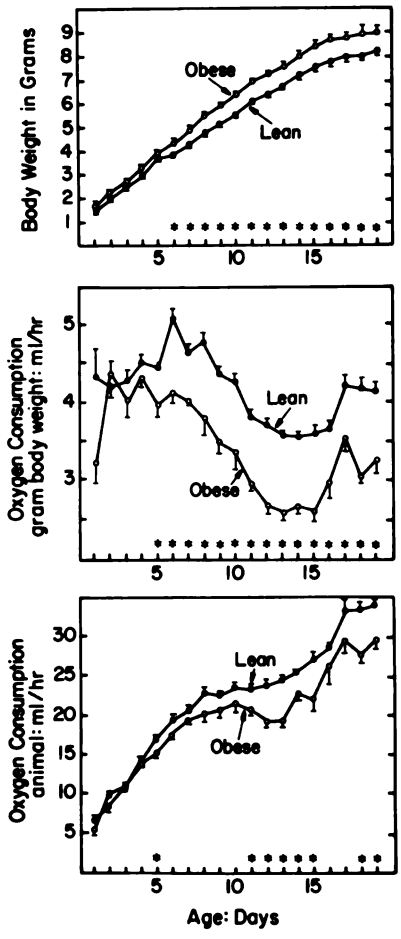


FIG. 1. Body weight and oxygen consumption of lean and obese mice from 1 to 19 days of age. Each point represents the mean ± SEM for 24–55 lean and for 8–27 obese mice, approximately equal numbers of males and females are represented. The asterisks indicate significant ($P < 0.05$) differences between lean and obese mice.

Lean and obese mice were identified at 7 and 14 days of age, on the basis of lower oxygen consumption per g body weight in obese mice, and the body fat content of these mice was determined (Table I). Thirty-one and 28 percent of the mice screened at 7 and 14 days of age, respectively, were classified as obese. This closely corresponds with the expected ratio of one obese to three lean from heterozygote crosses. Obese mice weighed more and consumed less oxygen per g body weight than did lean mice at both 1 and 2 weeks after birth. As early as 7 days after birth obese mice had 38% more fat than did lean mice; this difference increased to 53% at 14 days of age. At 21 days of age obese mice contain 100% more fat than lean mice (9).

A 25–50% decrease in oxygen consumption per g body weight has been reported in mice at the time of weaning (3, 4). Table II presents the oxygen consumption of mice several days before and after 21 days of age. Regardless of whether the pups remained with their mother or were weaned to a high-fat or a high-carbohydrate diet, oxygen consumption per g body weight decreased approximately 17% between days 18 and 20 and days 22 and 24 of age. However, oxygen consumption per animal was not changed during this period

TABLE I. BODY WEIGHT, OXYGEN CONSUMPTION AND BODY FAT CONTENT OF ONE AND TWO WEEK OLD LEAN AND OBESE MICE.[a]

	Age			
	7 days		14 days	
Parameter	Lean	Obese	Lean	Obese
Body weight:g	3.62 ± 0.10	4.34 ± 0.13[c]	7.09 ± 0.15	7.72 ± 0.16[c]
Oxygen consumption:ml/g/hr[b]	4.12 ± 0.08	3.30 ± 0.06[c]	3.52 ± 0.09	2.56 ± 0.05[c]
Body fat:%	6.4 ± 0.2	8.8 ± 0.2[c]	13.5 ± 0.4	20.7 ± 0.8[c]

[a] Mean ± SEM for 31 lean and 14 obese pups at 7 days of age and for 28 lean and 11 obese pups at 14 days of age, except body fat was determined on only 20 lean pups at 7 days of age. Approximately equal numbers of males and females were represented in each group.

[b] Oxygen consumption was determined on days 5–7 and on days 12–14; averages for the 3-day periods are presented.

[c] Significantly different from the lean value, $P < 0.05$.

TABLE II. OXYGEN CONSUMPTION OF LEAN MICE SEVERAL DAYS BEFORE AND AFTER 21 DAYS OF AGE.[a]

		Oxygen consumption	
Treatment	Parameter	Preweaned	Postweaned
Pups remained with mother	Body wt.-g	6.7 ± 0.05	7.8 ± 0.1[b]
	O_2-ml/g/hr	6.08 ± 0.18	5.09 ± 0.16[b]
	O_2-ml/animal/hr	40.53 ± 1.24	39.47 ± 1.30
Pups weaned to high-fat diet	Body wt.-g	6.8 ± 0.2	8.0 ± 0.2[b]
	O_2-ml/g/hr	5.98 ± 0.25	4.64 ± 0.18[b]
	O_2-ml/animal/hr	40.59 ± 2.00	36.22 ± 1.43
Pups weaned to high-carbohydrate diet	Body wt.-g	6.9 ± 0.1	8.4 ± 0.1[b]
	O_2-ml/g/hr	5.78 ± 0.25	4.97 ± 0.17[b]
	O_2-ml/animal/hr	39.88 ± 1.92	41.69 ± 1.63

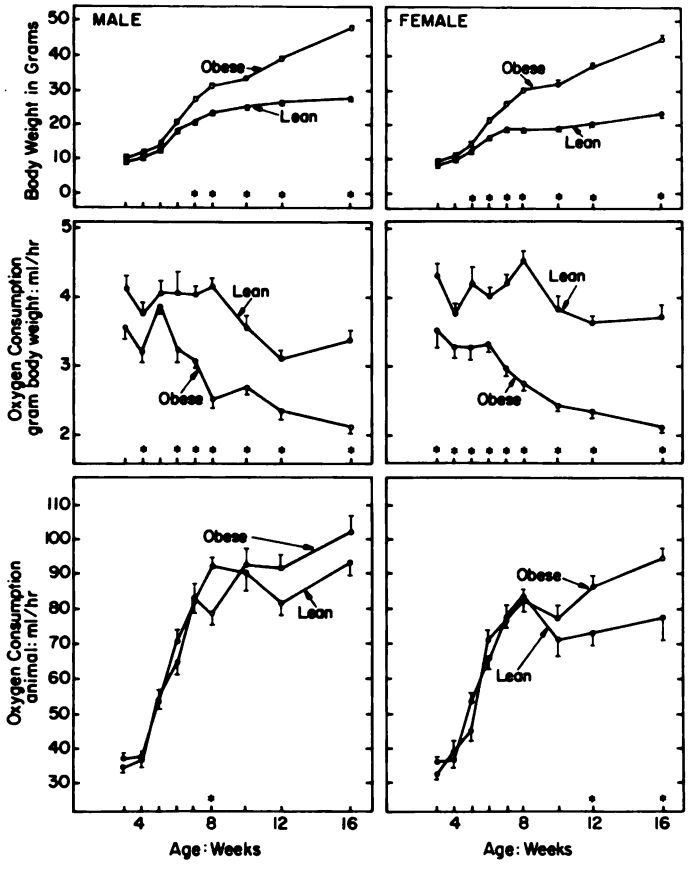
[a] Values are means ± SEM for 15–24 mice. There were five to six pups per litter. Pups were removed from the mother on day 21, except in the one treatment where pups remained with the mother throughout the study. Observations were made on days 18–20 and again on days 22–24. Approximately equal numbers of males and females were represented in each group.

[b] Significantly different from preweaned value, $P < 0.05$.

because body weight increased proportionately.

In the last experiment oxygen consumption rates of lean and obese mice were obtained from weaning to maturity (Fig. 2). Body weights of the obese mice were heavier than that of the lean mice; however, because of the variation within groups the differences were not statistically significant the first several weeks after weaning. Obese mice have been reported to be stressed more by weaning than are lean mice (9), which may have contributed to the variation in body weights during the first several weeks after weaning. Prior to weaning, body weights of the lean and obese mice were very uniform, this allowed small differences in body weight between the two groups to be statistically significant. Body weights of the obese mice continued to increase whereas body weights of the lean mice tended to plateau by 7–8 weeks of age. Oxygen consumption per g body weight of the obese mice was lower than that of the lean mice throughout the 13-week period, but the differences were not statistically significant at two of the points for male mice. Similar results were obtained when the rates were expressed per g to the 0.75 power. Oxygen consumption per animal increased two- to threefold for all animals. After 10 weeks of age obese mice tended to consume more oxygen per animal than did lean mice; the differences were statistically significant for the female mice.

Discussion. The results of this study clearly demonstrate that there are differences between lean and obese mice in body composition and energy metabolism as early as 1 week after birth. Previously, body fat content of obese mice had been reported to be elevated by 17–21 days of age (7, 9) and fat cell size of the obese mice to be increased above



2. Body weight and oxygen consumption of lean and obese male and female mice from 3 to 16 weeks of ch point represents the mean ± SEM for 10–26 lean and 11–16 obese mice. The asterisks indicate significant .05) differences between lean and obese mice.

vels observed in lean animals by 12–14 of age (5). Because of the magnitude of ifference in body fat between lean and pups at 7 days of age it is probable that ences in body fat already exist within a lays after birth. Hyperphagia does not ır to be a factor in the increased accu- tion of body fat in 1- to 3-week old obese (9).

e lower rectal temperature of obese pups days of age (6) and the altered response : obese pups to cold by 12 days of age ıggested that metabolic rates might be ed in these obese mice prior to the ɔusly observed depressions at 17–20 ɔf age (2–4). In the present study differ- between lean and obese mice in oxygen mption per g body weight were ob- l by the 5th day of age. Several days later the obese mice exhibited an increased body fat content.

All oxygen consumption measurements were recorded at 30° in the present study; this temperature is probably below the thermoneutral zone for the very young mice (14). The changes in oxygen consumption per g body weight from 1 to 19 days of age may reflect in part a change in the thermoneutral zone of these young mice with development. It has been reported that differences in oxygen consumption between young obese and lean mice were easier to detect as the temperature of the determination was increased from 20° to 25° (3, 4). Whether a shift in temperature would allow demonstration of differences in oxygen consumption between lean and obese mice prior to 5 days of age was not determined.

Oxygen consumption of lean mice per g body weight decreased approximately 17% between days 18 and 20 and days 22 and 24 of age regardless of the treatment imposed. These measurements were conducted at 30°. The decrease in oxygen consumption was approximately 24% at 25° (4) and 28% at 20° (3). The greater drop in oxygen consumption observed at the lower ambient temperatures may reflect a lowering of the thermoneutral zone of these mice as they develop (14). A decrease in energy intake decreases metabolic rate, at least in certain species (13, 15). Energy intake of these mice has been suggested to be decreased for several days immediately after weaning (9). Mice left with their mother may also have undergone similar changes in food intake because milk production decreases markedly after the 20th day of lactation (9). These factors together with the general decrease in oxygen consumption per unit weight as body weight increases (16) probably explain the previously observed changes in oxygen consumption (3, 4) at the time of weaning.

Beyond 3 weeks of age oxygen consumption of obese mice, expressed per animal, was very similar to that observed in lean mice even though obese mice were heavier and consumed more food than lean mice (1, 9). The mechanism(s) responsible for the increased energy efficiency of the obese mice remains to be established.

Summary. Rates of oxygen consumption were determined daily from birth to 19 days of age and weekly thereafter until 16 weeks of age in lean and obese mice. As early as 5 days after birth obese mice consumed less oxygen than lean mice. Obese mice weighed more than lean mice by 6 days of age and contained 38% more fat than lean mice at 7 days of age. At 14 days of age obese mice contained 53% more fat than lean mice. Beyond 3 weeks of age oxygen consumption of obese mice was less than observed in lean mice when the results were expressed per g body weight, but the values for obese and lean mice were similar when expressed per animal. These results demonstrate that alterations in energy metabolism occur very early in the life of obese mice.

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Glucose Metabolism in Obese Men: Abnormal Intravenous and Normal Oral Glucose Tolerance (40064)

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Obesity and its insulin resistant state are well known to pose additional risk for the development of diabetes mellitus (1). With increasing evidence that even mild degrees of hyperglycemia may hasten the development of large vessel and microvascular disease in diabetes (2) earlier detection of diabetes has been encouraged.

In the past, several studies of glucose tolerance in obese women have shown apparent preservation of intravenous glucose tolerance, but rather consistent abnormalities in the handling of orally administered glucose (3, 4). In the course of reviewing glucose tolerance in obese men, however, we have found that the opposite obtains, i.e., grossly overweight, nondiabetic men are apparently capable of preserving oral glucose tolerance while results of intravenous testing revealed abnormal disposition of the administered glucose.

Methods and materials. Eleven markedly obese male subjects (mean weight 190 ± 13% of normal; range 142–278% of ideal body weight) (5) with no personal or family history of diabetes, were studied. Mean age at time of the investigation was 38 years (range 25–49 years). All the patients were ambulatory and in good health, and received no less than 250 g of carbohydrate daily in their hospital diet for 3 days prior to testing.

After an overnight fast, and with the patient at rest, an indwelling scalp vein needle was inserted in an antecubital vein, kept open with a slow drip of heparin and saline (5 U/cc). For oral glucose tolerance testing a dose of glucose of 1 g/kg of ideal body weight and dissolved to constitute a 30% solution, was consumed within 10 min, and venous blood samples were drawn at 30, 60, 90, 120 and 180 min. For intravenous glucose tolerance testing, and with several days between the above tests, the subjects received an intravenous glucose infusion of 0.5 g/kg/ideal body weight over a 3-min period. Baseline samples were drawn, as well at 5, 15, 25, 35, 45 and 55 min after beginning the infusion of glucose.

Plasma glucose was measured by the Technicon Auto Analyzer using the method of Hoffman (6). Serum immunoreactive insulin was determined by a modification of the dextran–charcoal method (7).

The results of the oral glucose tolerance tests were evaluated using the Fajans and Conn criterion (8, 9) (glucose levels not higher than 185 mg% at 1 hr, or 140 mg% at 2 hr), the sum oral GTT/2 hrs diagnostic standard (8, 10), (summation of fasting, 1 and 2 hr plasma glucose levels), and by the Wilkerson point system (11), (fasting and 3 hr, 130 mg%; 1 point each if higher; and 1 hr 195 mg%, 2 hr 140 mg%; half point each if higher) All values are referred to measurements of glucose in plasma, which are higher than in whole blood (11).

Intravenous glucose tolerance was expressed as a function of the disappearance rate of glucose, obtained by plotting absolute glucose values semilogarithmically against time (12).

Results. Despite gross obesity each of these male subjects demonstrated oral glucose tolerance considered to be within limits by the sum of glucose levels for 2 hr (normal less than 450 mg) (8), by the Wilkerson point criteria (normal 1.5 points or less) (11) or by the Fajans and Conn criteria (normals show no more than one abnormal value at either 60 or at 120 min (8, 9) (Table I).

Despite normal oral glucose tolerance, all subjects but one displayed abnormal disappearance rates ($k < 1.20$) and in at least one third of the subjects the disappearance rates were clearly diabetic ($k = 0.9$ or less). Only one intravenous glucose tolerance was unequivocally normal ($k > 1.20$), (Table I, subject No. 11), (12).

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TABLE I

Patient No.	Weight percentile (of ideal) %	Oral glucose tolerance 2 hr SUM	Oral glucose tolerance 60 min. (mg%)	Oral glucose tolerance 120 min. (mg%)	Points[a] (Wilkerson)	Intravenous glucose tolerance *K* values
1	196	358	162	116	—	0.64
2	278	396	166	136	—	0.88
3	170	311	126	100	—	0.89
4	167	339	145	126	—	0.89
5	185	358	151	119	—	0.92
6	245	388	160	130	—	0.98
7	155	378	140	158	0.5	1.00
8	155	386	171	132	—	1.07
9	220	396	168	148	0.5	1.14
10	142	341	153	123	—	1.17
11	181	328	139	122	—	1.36
Mean	190	362	153	128	—	0.994
SEM	12.6	8.8	4.3	4.7	—	0.056

[a] All fasting values were below 110 mg%, and at 180 min were below 125 mg%.

No correlations were found between the summation of glucose levels for 2 hr during glucose tolerance testing (2 hr sum/GTT) (8) and the "*k*" values representing intravenous glucose disposal rate ($r = 0.0373$, p = not significant). Furthermore, no correlation could be established between the 2-hr glucose value from the oral study and disappearance rates following intravenous glucose administration ($r = 0.2108$, p = n.s.). Finally, although no significant correlation could be established between excess percentile body weight and abnormal disposition of an intravenously administered glucose load ($r = 0.231$), there was a perceptible tendency towards lower "*k*" values in the more obese patients (Table I).

When these obese men were divided into those with "*k*" values below 0.9 (clearly abnormal) and above 0.9 (borderline or normal) no significant differences were noted in either oral glucose tolerance values or serum insulin levels during both tests (Fig. 1).

However, it may be observed that the significant difference (by definition) between mean glucose values in the intravenous glucose tolerance tests, ($P < 0.025$) appear reversed, although nonsignificantly, during the oral tests. Also, higher mean insulin levels were perceptible after both the oral and intravenous glucose challenges in the group with abnormal intravenous glucose tolerance tests (Fig. 1B).

Discussion. Reports of the ability to maintain normal disposal of an intravenously administered glucose load in the face of impaired oral glucose tolerance in nondiabetic obese women (3, 4) has been associated with earlier suggestions that hepatic glycogenesis is decreased in obesity, (14) or that a rising blood sugar fails to normally dampen hepatic glucose output (3). By contrast, however, this study of oral and intravenous glucose tolerance in obese males revealed the opposite. Intravenous glucose tolerance, rather than orally administered glucose, seemed to un-

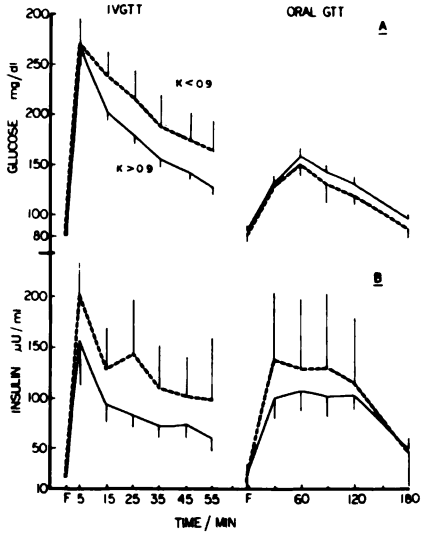

FIG. 1. A. Upper panel: Mean plasma glucose values (± SEM) during intravenous (IVGTT) and oral glucose tolerance tests (oral GTT) in obese subjects. B. Lower panel: Mean serum insulin levels (± SEM) during the same tests. Full line: patients with IVGTT disappearance rates (*K*) higher than 0.9. Interrupted line: IVGTT *K* values lower than 0.9.

ulin resistance although handling of
lly administered glucose load re-
intact. Insulin release for those men
impaired intravenous glucose toler-
is actually greater than for those
he group who maintained normal
: to both parenteral and oral routes
se administration. This increased in-
ease was insufficient to maintain nor-
osition of the intravenously admin-
load, but the augmented insulin
luring the oral study may have more
ely insulinized the hepatocyte
more efficiently promoting decreased
iic glucose output in these subjects
the other hand, the intravenous glu-
rance test, rather than the oral test,
ave evidenced peripheral resistance
se utilization (15), since a lower pro-
of an intravenous glucose load is
handled by the liver (16, 17).

se these results differ so from what
reported in overweight women, (3,
y also be that there is a sex-related
e in disposal of intravenously ad-
ed glucose in obese subjects. The high
e of impaired intravenous glucose
in these obese men, reflecting
peripheral resistance to the biologic
endogenously released insulin, might
gest a difference on the basis of sex.
be noted, however, that these obese
re probably heavier than the previ-
blished accounts of intravenous and
icose tolerance testing in obese
only defined by body weights greater
% (3) and 120% (4). This fact alone
count for greater peripheral insulin
e and the results of this study.

nformation from this study amplifies
tion should be exercised when at-
to accurately interpret the results of
tolerance testing. Both oral (18, 19)
avenous (20) glucose tolerance tests
en shown to lack reproducibility or
bility and several reports have ac-
monstrated that there can be total
greement between the results of both
intravenous glucose tolerance testing
me subject on different days (20–22).
ethods for investigating glucose dis-
) have also failed to correlate exactly
results of intravenous glucose toler-
ing, although a positive correlation
with the results of glucose by mouth has been reported from studies where prolonged intravenous infusion of glucose was used (23).

Although it would be desirable to confidently quantitate the risk of becoming diabetic from the results of glucose tolerance testing, the data derived in our obese male population mostly suggest that, in the male subject, intravenous rather than oral glucose tolerance testing might reveal earlier deterioration. Whether abnormal handling of parenterally administered glucose bears any real relation to added risk for actually developing clinically significant diabetes mellitus remains to be established. Nevertheless, these results do raise the interesting possibility that there may also be a sex-related difference in hepatic glycogenesis and gluconeogenesis which bears further investigation.

Summary. Previous reports from moderately obese women have demonstrated that while intravenous glucose tolerance is generally preserved, oral glucose tolerance is frequently abnormal. On the basis of such findings it has been suggested that oral glucose tolerance testing would more reliably predict or identify the truly latent diabetic obese. In an effort to reassess this proposition, we have administered oral glucose loads and intravenous glucose challenges to 11 severely obese, otherwise healthy, male subjects. Contrary to the reports in women, intravenous glucose tolerance was abnormal or borderline in 10 of 11 men in this group, whereas oral glucose tolerance was normal by three current criteria in all subjects studied. Insulin levels rose equally high in both tests and did not correlate with these discrepant results. As a lower proportion of an intravenous glucose load is directly handled by the liver, and others have reported that hepatic resistance to the action of insulin can be reversed in the presence of high insulin levels in obesity, it is suggested that intravenous administration of glucose would unmask peripheral insulin resistance in the obese male subject.

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ɔls of Blood Group Synthetic Enzymes in Human Colonic Carcinoma[1] (40065)

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lorectal carcinoma, the reappearance noembryonic antigen (1) and the loss n specific antigen (2, 3) suggest a associated with dedifferentiation of ignant cells. Loss of ABH isoantigen served in gastrointestinal carcinoma s postulated to be due to a similar ism (4). In this study we report on the of enzymes responsible for the syn- f A or B isoantigen in the colorectal us tissues and its adjacent normal tis- eliminary results of this study were sly presented (5).

rials and methods. Eighteen patients lorectal carcinoma were included in dy. Seven patients belonged to blood O); 6 (A); 3 (B); and 2 (AB). The ı of tumor varied from cecum to rec- ıble I). Normal and cancerous colonic were frozen immediately in dry ice ırgical removal and stored at −80° sed for assay. Colonic mucosa was d by scraping the mucosal layer from p of resected colon. The normal mu- yer was obtained from the tissues at :m away from the tumor in the tumor- ırgin. At that distance the tissue was abnormal cells as judged by micro- :xamination.

-D-galactose:2′-fucosyllactose galac- ınsferase (Gal transferase) and UDP- yl-D-galactosamine:2′-fucosyllactose yl-galactosaminyl transferase (Gal- ınsferase) activities were measured by hod of Kobata, Grollman and Gins-). Approximately 100 mg of tissue ɔmogenized in 1 ml of 0.05 *M* Tris, *M* mercaptoethanol (pH 6.8) at 0°. :ntrifugation at 1000*g* for 15 min, 0.1 ml of supernatant was incubated with 16 m*M* Tris–Cl pH 7.5, 8 m*M* $MnCl_2$, 0.24 mM 2′-fucosyl lactose [O-α-L-fucopyranosyl-(1 → 2)-O-β-D-galactopyranosyl-(1 → 4)-D-glucose] (7), 0.24 m*M* UDP-D-galactose (galactose ^{14}C[G] 0.4 Ci/mole) or UDP-*N*-acetyl-D-galactosamine (galactosamine-1-[^{14}C], 1.5 Ci/mole) (both from New England Nuclear, Boston) (total aqueous volumne 125 μl) and 25 μl toluene at 37° for 20 h. The time course of the sugar incorporations was nonlinear from the outset in the cases where the entire time course was studied. The ratio of incorporation (tumor/normal) was essentially constant from the beginning to the end. We chose the long incubation time to be certain that very low levels of activity would not be overlooked. In fact, the sugar incorporation at 4 hr was about 80% of that at 20 hr. Under the conditions of the assay, the amount of product formed was proportional to the amount of tissue homogenate used. At the end of the incubation, the mixture was heated at 100° for 3 min and applied to a 1 ml column of Dowex-1 × 8-Cl previously treated by washing with 0.02 *M* lactose followed by water. This treatment reduces the nonspecific absorption of the neutral sugar products. The unabsorbed products were washed from the columns with 5 ml H_2O and the eluates concentrated to dryness *in vacuo* at 30°. The residue was dissolved in 100 μl of water, spotted on Whatman 3 mm paper and chromatographed for 30 hr using ethyl acetate–pyridine–H_2O (2:1:2) as the solvent. After drying, the chromatogram was scanned for radioactivity. The magnitude of radioactivity in the tetrasaccharide peak was estimated by graphical integration with the resulting value converted into micromoles of galactose or galactosamine incorporated per gram of tissue in 20 hr using appropriate standards.

Three control systems, one without tissue homogenate, one with homogenate which had been heated to inactivate the enzyme and

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TABLE I. SITE OF COLORECTAL CANCER IN 18 PATIENTS STUDIED.

Sites of cancer	Patient study numbers
Cecum	20
Ascending	4
Transverse	1, 7, 9
Descending	13
Sigmoid	8, 16, 17, 19, 21
Rectosigmoid	5, 12, 14
Rectal	2, 3, 6, 22

a third without 2′-fucosyl-lactose, were included and performed simultaneously in each experiment. The enzyme activities were also examined in whole blood samples from normal individuals.

The α-galactosidase activity, a possible degradative enzyme for B substance, was measured by the method of Levvy and Conchie (8), using *P*-nitrophenol-α-D-galactopyranoside as the substrate.

Results. The radioactive "tetrasaccharide" peak, which was not found in the three control systems as described in the previous section, was observed in the complete assay system (Fig. 1). The tetrasaccharide peak was further characterized to be α-D-galactosyl 2′-fucosyllactose by graded acid hydrolysis and coffee bean α-galactosidase to yield the appropriate mono- and oligosaccharides.

The activities of Gal transferase in normal and tumor tissues are shown in Table II. The transferase was found in tissues from patients of blood group B or AB. Four (No. 16, 17, 1 and 7) of five tumors had increased activity in cancerous tissue by 1.6- to 6.9-fold. The normal or tumor tissues of group A or O did not contain this enzyme.

Nonspecific α-galactosidase activities were studied in nine tumors. They were elevated by 1.3- to 2.7-fold in A, B, and AB tumors, and decreased in three of four group 0 tumors. When preformed tetrasaccharide from the biosynthetic experiments of B tumor were incubated with A or B tumor homogenate, there was no galactose released. This suggests that the nonspecific α-D-galactosidase in tissue can not hydrolyze galactosyl fucosyllactose and thus does not influence the result of α-D-galactosyl transferase measurements *in vitro*. The activity of galactosyl transferase in AB or B blood was negligible at the level of 0.048 and 0.061 μmoles/ml of blood in 20 hr respectively.

The activity of GalNAc transferase was found only in normal or cancerous colorectal tissues from patients with blood group A or AB (Table III). The tumor/normal ratio varied widely from patient to patient

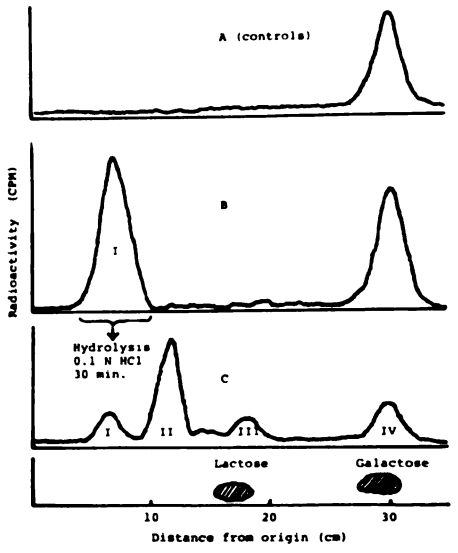

FIG. 1. Chromatography of radioactive oligosaccharides formed by a galactosyl transferase of human colonic cancer tissue. The formation of tetrasaccharide peak (I) was found after incubation of tissue homogenate with UDP-D-Gal-[^{14}C] and its acceptor fucosyllactose (Graph B); Peak (I) was not observed in three sets of control, one without tissue homogenate, one without fucosyllactose, and the third with heated tissue homogenate (Graph A); when the Peak I in graph B was subjected to acid hydrolysis and rechromatographed, Tri-, di-, and monosaccharides were found (Peaks II, III and IV in Graph C).

TABLE II. α-D-GALACTOSYL TRANSFERASE ACTIVITIES IN NORMAL AND CANCEROUS COLON.

Blood group	Patient study number	UDP-Gal transferase activities μmoles/g tissue/20 hr		
		Normal	Tumor	Ratio (tumor/normal)
B	8	0.70	0.07[a]	—
	16	0.99	6.85	6.92
	17	2.55	4.70	1.84
AB	1	0.38	1.08	2.84
	1 (repeated)	(0.32)	(1.06)	(3.31)
	7	0.29	0.45	1.55
A	3, 9, 12, 14, 20, 22	0	0	—
O	2, 4, 5, 6, 13, 19, 21	0	0	—

[a] Necrotic tumor.

LE III. Gal NAc TRANSFERASE ACTIVITY IN NORMAL AND CANCEROUS COLON.

Patient study number	UDP-Gal NAc Transferase Activity μmoles/g tissue/20 hr		
	Normal	Tumor	Ratio (tumor/normal)
9	0.073	0.052	0.71
12	0.438	0.037	0.08
14	0.279	0.300	3.58
20	0.025	0.337	13.5
1	0.014	0.071	5.07
1 (repeated)	(0.017)	(0.059)	(3.47)
7	0.156	0.115	0.74
8	0	0	
2, 4, 5, 6, 13	0	0	

13.48). The enzyme activity in cancer-
ssue increased in three of the six pa-

cussion. The levels of galactosyl trans-
s have previously been studied in var-
issues from patients with intestinal tu-
(9–11). Galactosyl transferase isoen-
II was detected in serum in 13 of 17
its with colorectal adenocarcinoma, but
bserved only in two of 39 patients with
alignant gastrointestinal disorders (9).
ntration of galactosyl transferase II de-
d in colonic cancerous tissue (10), but
ised in tumors of small intestine (11). It
d be noted that the substrate used for
erase assay was not 2′-fucosyllactose,
he serum or tumor tissues were from
group-nonspecified patients; therefore,
izymatic activity may not represent the
group B specific enzyme, α-D-galacto-
nsferase. The methods used for deter-
ion of the activity of Gal transferase or
Ac transferase in this study were blood
specific, since Group B synthetic en-
was not detected in Group A or O
its and vice versa.

variation in transferase activities
g the normal tissues was large but not
ected. Using 2′-fucosyllactose as the
tor, Kobata and Ginsburg found a fif-
ld range in the *N*-acetyl-D-galactosa-
transferase activities in the milk of type
men (12). Race *et al.* found an eleven-
variation in the galactosyl transferase
ty in the stomach mucosa of type B
ns using the same acceptor (13). It is
n that antigenic properties varied
g subgroups of type A. Activity of α-GalNAc transferases in sera of A_1 human subjects was considerably higher than that of A_2 (14). In a Caucasian population of blood donors with normal B phenotype, 16% of population has α-D-galactosyl transferase activity 1.94 times higher than the rest of the population (15). These may reflect the variant gene expression. Because of this wide variation of blood group related transferase activities in normal colon tissue, the activities in cancerous tissue were expressed as tumor/normal ratio in each individual. Tumors from types B or AB patients invariably had higher galactosyl transferase activities than adjacent non-tumorous tissues. In one-half (3 of 6) of the patients with A or AB blood group, the level of acetyl-D-galactosamine transferase was increased in the tumor. It is conceivable that the higher density of epithelial cells in the tumor tissue may be responsible for the increased activity. When the results were re-stated on the basis of activity per mg of protein in the homogenate, there was no qualitative difference in the pattern of enzyme activity changes.

The possibility of bacterial contamination as the cause of increased B antigen should be considered. Marsh has found a factor in a bacterial filtrate which produced B antigens in O and A red cells *in vitro* (16). Since galactosyl transferase activity was never found in either normal or cancerous tissues from 13 patients with blood group A or O, the possibility is highly remote that any of the enzyme activity found in B or AB tissue might be derived from bacterial contamination.

From the results of this study, it is apparent that loss of ABH isoantigens in colorectal cancer as documented in fluorescein-conjugated antiserum technique (4), can not be ascribed to a general lack of A or B isoantigen synthetic enzymes. It was recently demonstrated that human feces contain enzymes produced by enteric bacteria that degrade the A, B, and H blood group antigens (17). However, it will be difficult to explain why the bacterial-producing enzymes should act differently between normal and cancerous tissues.

Although we have documented that the transferases involved in A or B isoantigen synthesis are not deficient in cancerous tissue, there could still be a defect on the synthetic

pathway prior to transferase step leading to a loss in the ABH isoantigens. However, a more likely reason in our opinion is that the changes are due to an accumulation of mucoprotein in the colonic cancer (18) which may mask the expression of surface antigen(s) (19) and cause a negative result for the immunofluorescent stain technique for detecting ABH isoantigen. In leukemic patients, loss of blood group antigen in red blood cells has also been observed (20), but the expression of the antigen could be recovered with neuraminidase treatment (21).

Summary. Homogenates of tumorous and adjacent non-tumorous colorectal tissues from 18 patients were tested for the activities of blood group synthetic enzymes, namely α-D-galactosyl transferase for B isoantigen and α-*N*-acetyl-D-galactosamine transferase of A isoantigen.

The galactosyl transferase activity in non-tumorous intestinal tissue was high in patients with blood group B, intermediate in group AB and was absent in group A or O. As compared with adjacent non-tumorous tissue, the Gal transferase activity increased in tumors by 1.6- to 6.9-fold in four of five patients with blood group B or AB, and the α-*N*-acetyl-D-galactosaminyl transferase activity increased in three- of six-tumor tissues from patients of blood group A or AB.

The results suggest that the reported losses of ABH isoantigen in colorectal cancer are not due to deficiencies of Gal or GalNAc transferase activities involved in the synthesis of blood group antigens.

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Influence of Adrenalectomy and of Corticosterone Administration on the Ether-uced Increase in Plasma Prolactin in Ovariectomized Estrogen-Treated Rats[1] (40066)

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ıave reported that various anesthetics prolactin in ovariectomized (OVX) 'X–estrogen treated rats (1–3). A num-investigators have observed that pro-ınd corticosterone are both released by g (4–7) and stressful conditions (6–9). investigators have indicated that the stration of dexamethasone, a synthetic ɔrticoid, will attenuate the ether-in-(10, 11) and thyrotropin releasing hor-TRH)-induced (12) prolactin release. ɛsent study was undertaken to examine effect of continuous ether anesthesia r on prolactin release in adrenalecto-animals and (b) the influence of ex-ısly administered corticosterone, the -specific glucocorticoid in the rat, on ıduced prolactin release.

ɛrials and methods. Female .e–Dawley rats (Spartan Research An-Inc., Hasslett, MI) were ovariectomized when 200–225 g in body weight and under controlled temperature ') and artificial illumination (14 h light) h darkness). When they were about n body weight an aortic catheter was d as described in detail previously (1) ɛ rats were given a single subcutaneous ɔn of 1 mg Estradurin (equal to 0.5 mg 'estradiol phosphate). Bilateral adren-nies (ADX) were performed 3 days ıtheterization and ADX animals were ined on 0.9% NaCl. Serial blood sam-'as initiated 8 days after catheterization ıe experimental protocol was as de-l previously (3). Briefly, between 08:00 l 09:00 hr on the day of sampling, a ıylene extension filled with saline and 'itha three-way stop cock was attached indwelling catheter and the animals eft undisturbed for at least 60 min. After this equilibration period an initial blood sample (0.6 ml) was withdrawn (time 0). In the control group subsequent blood samples were obtained at 10, 30, 60, and 120 min. In the experimental groups, after the 0 min sample, the animals were anesthetized in a large jar saturated with ether vapor. In those animals receiving corticosterone, a single bolus of the hormone was administered intraarterially through the catheter after the 0 min sample. Complete anesthesia was achieved within about 2 min, and the rats were maintained under ether for the duration of the experiment using a nose cone. Additional blood samples were obtained at 10, 30, 60, and 120 min after the initiation of anesthesia. After each blood sample the volume was immediately replaced with sterile saline at 37°. The blood samples were diluted with an equal volume of chilled phosphate buffer saline (pH 7.6) and centrifuged at 3°. The plasma was separated and stored at −20°. Plasma prolactin levels were assayed by the double antibody radioimmunoassay (13) at two dilutions each in duplicate. Rat prolactin NIAMD-RP-1 with a potency of 11 IU/mg was the standard. Plasma corticosterone levels were determined by competitive binding assay (14) in duplicate at a single dilution. Statistical comparisons were made within a group using the analysis of variance and Duncan multiple range test and between treatment groups using a two-way analysis of variance with computor assistance (15).

Results. The plasma prolactin values are shown in Table I. The plasma prolactin levels in the control animals were not significantly altered by the sampling procedure. In the OVX–Estradurin-injected animals (OVX–E_2) under ether, there was a significant rise in plasma prolactin in 10 min ($P<0.01$), followed by a decrease to a slightly higher level than the initial value and this level was maintained for the remainder of the experiment

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(120 min). The OVX–ADX–E_2 animals under ether were observed to have an increase in plasma prolactin for all time periods when compared with the initial level. The administration of corticosterone (500 μg or 25 μg/rat i.a.) completely block the initial ether-induced prolactin increase (10 min); however, at subsequent time periods there was a gradual increase in plasma prolactin levels to that of adrenalectomized animals (120 min; Groups IV and V vs III).

The plasma corticosterone levels are presented in Table II. In the OVX–E_2 animals under ether, plasma corticosterone at 30, 60, and 120 min were significantly higher than the initial 0 min value. The OVX–ADX–E_2–corticosterone (500 μg/rat) administered animals had extremely high plasma corticosterone level at 10 min which progressively declined in subsequent periods. Those animals injected with 25 μg/rat of corticosterone showed a similar trend but with a less pronounced increase in plasma corticosterone at 10 min.

Discussion. In estrogenized animals a rise and decline in plasma prolactin following ether anesthesia has been observed in this study and in our previous studies (2, 3). This pattern of prolactin release was not restricted to only ether but has been observed with other anesthetics such as methoxyflurane (3) and with restraint stress (16). It has been reported by others that the prolactin increase to ether stress (10, 11) and TRH administration (12) could be attenuated by dexamethasone. Further, it has been shown that in situations like suckling, plasma corticosterone increased in conjunction with prolactin (7). The plasma prolactin pattern of a sustained increase under ether in ADX animals and a sharp rise and fall in adrenal intact animals suggests that, under ether, the fall in prolactin after the initial increase may be due to the inhibitory feedback action of the elevated corticosterone. This hypothesis was further strengthened by the results obtained in this study. Here following exogenous administration of corticosterone there was no increase in plasma prolactin following ether anesthesia; however, as the exogenous corticosterone was metabolized from circulation, the plasma prolactin level increased. Although previous studies with dexamethasone suggested a negative feedback (10–12), the use of corticosterone, which is a natural glucocorticoid, lends further credence to the hypothesis that adrenal corticoids have an inhibitory effect on prolactin secretion in rats. This inhibitory feedback action, however, does not appear applicable to all conditions of increased prolactin release since in suckling, where prolactin release is more pronounced and sustained (6, 17) than during ether exposure (6), and where there is also a simultaneous increase in plasma corticosterone levels (7), little to no decrease in plasma prolactin is observed. This suggests that in rats there may be a different mechanism between suckling and ether in the induction of prolactin release.

Summary. Ovariectomized, polyestradiol-phosphate (PEP) injected, catheterized rats subjected to continuous ether anesthesia

TABLE I. EFFECTS OF ETHER ANESTHESIA ON PLASMA PROLACTIN LEVELS.

Group	Treatment	Plasma prolactin levels (ng/ml)**				
		0 min	10 min	30 min	60 min	120 min
I	Control OVX + E_2 (7)*	59.5 ± 11.1	50.7 ± 5.3	60.4 ± 8.4	48.5 ± 7.8	55.6 ± 4.6
				under ether anesthesia		
II	OVX + E_2 + Ether (7)	44.5 ± 3.6	108.2 ± 17.1[b]	68.1 ± 10.5	64.7 ± 15.7	67.2 ± 14.2
III	OVX + ADX + E_2 + Ether (15)	46.0 ± 4.3	120.3 ± 23.5[a]	109.0 ± 18.6[a]	125.7 ± 22.7[b]	111.9 ± 21.0[a]
IV	OVX + ADX + E_2 + B 500 μg/rat + Ether (10)	43.0 ± 2.8	64.1 ± 14.6	74.2 ± 9.7	87.9 ± 15.9	99.3 ± 25.0
V	OVX + ADX + E_2 + B 25 μg/rat + Ether (5)	44.4 ± 6.9	42.4 ± 3.9	95.6 ± 20.4	59.0 ± 6.3	126.4 ± 43.0

* The number in parentheses represents the number of animals in each group. Statistical comparisons were made using analysis of variance and comparisons of each time period were made to the 0 min values within each experimental group using Duncan's multiple range test (a = $P < 0.05$, b = $P < 0.01$). Using two-way analysis of variance the following group comparisons for treatments were made: I vs II, NS; II vs III, $P < 0.05$; III vs IV $P < 0.05$; III vs V, NS. OVX = ovariectomy; ADX = adrenalectomy; E_2 = polyestradiol phosphate; B = corticosterone.

** Mean ± SE.

TABLE II. EFFECTS OF ETHER ANESTHESIA ON PLASMA CORTICOSTERONE LEVELS.

up	Treatment	Plasma corticosterone levels (μg/100 ml)**				
		0 min	10 min	30 min	60 min	120 min
	Control OVX + E_2 (7)*	34.8 ± 5.4	45.2 ± 5.8	50.5 ± 3.5	50.2 ± 10.4	36.2 ± 8.5
				under ether anesthesia		
	OVX + E_2 + Ether (7)	34.0 ± 3.7	53.4 ± 6.7	59.7 ± 7.7[a]	63.4 ± 6.6[b]	62.7 ± 8.4[a]
	OVX + ADX + E_2 + B 500 μg/rat + Ether (10)	6.3 ± 0.6	2110.0 ± 224.0[b]	202.3 ± 16.8[b]	56.7 ± 8.6[b]	18.3 ± 3.5[b]
	OVX + ADX + E_2 + B 25 μg/rat + Ether (5)	6.0 ± 0.5	161.0 ± 24.7[b]	26.0 ± 4.3[b]	7.6 ± 0.4	5.8 ± 0.6

The number in parentheses represents the number of animals in each group.
* Mean ± SE.
tatistical comparisons were made using analysis of variance and comparisons of each time period were made to) min value within each experimental group using Duncan's multiple range test (a = $P < 0.05$; b = $P < 0.01$). g two-way analysis of variance the following group comparisons for treatments were made: I vs II, $P < 0.05$. (= ovariectomy; ADX = adrenalectomy; E_2 = polyestradiol phosphate; B = corticosterone.

ved an increase in plasma prolactin level 0 min followed by lower levels at 30, 60, 120 min. Plasma corticosterone levels e increased significantly under continuous :r anesthesia. In contrast to intact animal, terally adrenalectomized animals under :r anesthesia showed a sustained elevation)lasma prolactin at all time periods. In :nalectomized animals subjected to ether ;thesia the exogenous administration of icosterone (either 500 μg/rat or 25 μg/rat) /ented the increase in plasma prolactin at ıin period when plasma level of corticos- ne was high. In subsequent periods as the ma corticosterone decreased, the plasma actin increased. It is suggested that cor- sterone has an inhibitory feedback effect he ether-induced prolactin release.

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Correlation of Chromium Sesquioxide and [^{14}C]Cellulose as Fecal Markers in Rats (40067)

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The gastrointestinal transit time in a subject can be measured by having the subject swallow a nonabsorbable marker and measuring the time it takes for the marker to appear in the feces. Laboratory animals will not voluntarily swallow a marker such as barium impregnated polythene pellets (1), so, an investigator wishing to measure transit time in a laboratory animal must either force feed the marker or add the marker to a portion of the animal's food. Chromium sesquioxide is a marker which can be added to a diet without making it unpalatable. It is not absorbed (2), and it can be accurately measured. A marker should travel at the same rate as food residues, however, direct proof that the marker and the food residue travel at the same rate is difficult to obtain (1). This experiment was undertaken to determine whether chromium sesquioxide travels at the same rate as [^{14}C]cellulose through the gastrointestinal tract of laboratory rats. Cellulose is not digestible by mammalian enzymes and is not absorbed by rats in which coprophagy has been prevented (3).

Material and methods. Twelve male Sprague–Dawley rats (469–578 g), individually caged, were used for the experiment. The basal diet consisted of 64.5% corn starch, 20% casein, 10% corn oil, 4.5% mineral mix, and 1% vitamin mix to which 2.5%, 5%, 10%, or 20% nonlabeled cellulose was added. The diets were fed *ad libitum*. The marked diet was prepared by mixing chromium sesquioxide (J. T. Baker Chem. Co.), and about 4 mg of uniformly labeled [^{14}C]cellulose (ICN Radiochemicals) to some oil-free 2.5% cellulose diet. The [^{14}C]cellulose was prepared from *Canna indica* and is free from any soluble carbohydrates, hemicelluloses, pectin, and lignin. The corn oil was added after the two markers were uniformly mixed with the diet. The completed marked diet contained 0.42% chromium sesquioxide and 86,480 disintegrations per minute (dpm) of [^{14}C] activity per g of diet. The rats were randomly assigned to one of four blocks. A block of rats was assigned to each cellulose level for 3 days. On the third day the diet was removed at 6:00 PM. The rats were presented with a 1 g pulse of marked diet at 9:40 PM and any uneaten marked diet was removed at 10:00 PM and weighed. After the marked diet was removed, the diet of the previous 2 days was again supplied *ad libitum* to the rats. An hour after the marked diet was given, the rats were tail cupped (5) to prevent coprophagy and to collect the feces. Eight hours after giving the marked diet, the tail cups were removed and all feces from each animal were collected. After 3 days on any cellulose level, the block of rats was assigned to another cellulose level, until all the rats had been on all four levels of cellulose. It has been established by other investigators (3) that 98.6% of the [^{14}C]cellulose is passed in 3 days. The 8 hr fecal collection was dried at 100° and weighed. Each animal's feces were then ground to a powder and mixed. Each fecal collection was analyzed for chromium and [^{14}C] activity. Chromium in the feces was assayed by atomic absorption spectrophotometry after digestion of the organic matter with nitric acid and oxidation of Cr_2O_3 to Cr_2O_7 with perchloric acid (4). A sample of feces from each 8-hr collection was ignited and the CO_2 collected in a Packard 306 Oxidizer. The [^{14}C] activity was measured with a liquid scintillation counter.

The total dpm of [^{14}C] and μg Cr passed in 8 hr were determined and the percentage of the ingested markers passed was calculated. The linear regression of per cent [^{14}C] passed on per cent Cr passed, the correlation coefficient, and the 95% confidence interval were calculated. Additionally the partial correlation of dpm of [^{14}C] passed on μg Cr passed was calculated to remove the effect of variation in g fecal dry matter (8).

Results. The regression of per cent [^{14}C]

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d on per cent Cr passed yielded a cor-
on coefficient (r) of 0.97 (Fig. 1) which
t significantly different from 1.00 (P
)1, d.f.=46). The 95% confidence inter-
ır the slope (a) of this line is $0.90 \leq a \leq$
and the interval for the y-intercept is
$' \leq b \leq 1.70$.
e partial correlation of [^{14}C] on Cr re-
s the effect of variation in fecal dry
ır from the correlation of [^{14}C] and Cr.
partial correlation yielded an r of 0.97
ı is not significantly different from 1.00
0.001) (Fig. 2). The range in marker
tion appeared to be due primarily to
idual animal differences and not neces-
related to the cellulose content of the

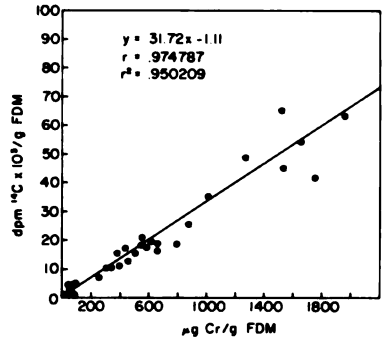
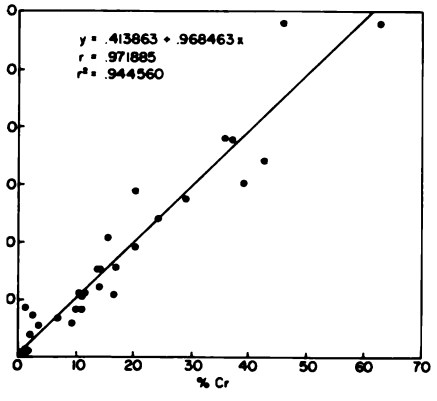


FIG. 2. Micrograms of Cr versus dpm of [^{14}C] from [^{14}C]cellulose passed in feces of tail-cupped rats during 8 h (n=48). Values are expressed per g fecal dry matter (FDM) passed in the 8-hr period.

cussion. These results demonstrate that
assage of [^{14}C] from cellulose in the feces
well correlated with the passage of Cr
chromium sesquioxide. The correlation
en these two fecal markers was inde-
nt from their correlation to the passage
al dry matter (Fig. 2).
er cent Cr passed is a good estimate of
nt [^{14}C]cellulose passed, the true regres-
ine should pass through the origin with
e equal to 1.00. Such a regression line
luded in the 95% confidence limits cal-
ed from these data indicating that per
Cr passed is a good estimate of per cent
passed in the feces. If cellulose were
n down in the gut some of the [^{14}C]
have been expired resulting in a low
recovery. However about 98% of [^{14}C]
ed cellulose can be recovered from the



. 1. Percent of dietary dose of chromium ses-
de and of dpm of [^{14}C] from [^{14}C]cellulose passed
s of tail-cupped rats during 8 h (n=48).

feces in tail-cupped rats (3). The specific gravity of chromium sesquioxide is 5.21 which is higher than the specific gravity of cellulose (1.27–1.61). Despite this difference our results indicate that chromium sesquioxide can be used to mark the portion of the gut contents in rats which contain cellulose. This is in contrast to the work of Kirwan and Smith (6) who found that increasing the specific gravity of a 10 mm by 4 mm capsule from 1.1 to 1.35 accelerated its passage through the gastrointestinal tract of humans, suggesting that the specific gravity of the marker would alter its rate of transit. Additionally, this study does not provide evidence that chromium sesquioxide had a tendency to settle out relative to the cellulose residue in the gut as suggested by Mitchell and Eastwood (7).

Summary. Tail-cupped rats were fed diets of varying cellulose contents. At the end of a 3-day period rats were given a pulse dose of a diet labeled with [^{14}C]cellulose and chromium sesquioxide. The results demonstrate that passage of chromium sesquioxide was highly correlated with passage of [^{14}C]cellulose and this correlation was independent of either marker's correlation with fecal dry matter. These results indicate that use of chromium sesquioxide as a fecal marker is a good estimate of cellulose passage in the tail-cupped rat.

The authors are grateful for the statistical assistance of Paul Schneeman.

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NGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 421–423 (1978)

ar Antibody Synthesis in Thiamin, Riboflavin, Biotin and Folic Acid-Deficient Rats[1] (40068)

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reased serum antibody titers in re- to a variety of antigenic stimuli have noted in a number of vitamin deficiency (1). In view of the complexity of factors ed in the establishment of serum anti- levels (2, 3), it is apparent that a more approach is required for elucidation of sic role of these vitamins in mediating immune responses. Investigation of an- synthesis at the cellular level affords an approach. Accordingly, we under- a series of studies on the effects of dual deficiencies upon the number of dy-forming cells in the spleens of im- ed rats. Our studies, to date, have dem- ted that the decreased serum antibody ses observed in vitamin B_6 and pan- ic acid-deficient rats can be attributed marked reduction in the number of dual antibody-forming cells in the s of these immunized deficient animals

iciencies of thiamin, riboflavin, biotin olic acid affect serum antibody re- s in the rat to varying degrees (6–10). s paper, we report the effects of these c deficiency states upon the develop- of splenic antibody-forming cells in im- ed rats.

terials and methods. Animals and diets. weanling, 21-day-old rats of the Holtz- strain were used. The animals were d individually in wide-meshed, screen- n, suspended cages and weighed y. Rats in the deficient groups were fed *itum* the basal, semipurified diets pre- y utilized for production of thiamin, vin, biotin and folic acid deficiencies (9). Control animals received the basal diet fed to the thiamin and riboflavin-deficient groups. In addition, each rat received daily a vitamin pill which, for control animals, supplied adequate quantities of the B-vitamins (9). For each deficient group, the corresponding vitamin was omitted from the pill. Rats in the riboflavin and biotin-deficient groups were fed the basal diets for 8–12 weeks prior to immunization. Corresponding periods for the folic acid and thiamin-deficient groups were 12–14 and 2–3 weeks, respectively.

Antigen. Sheep erythrocytes served as antigen in this study. Red blood cells were obtained commercially[3] in Alsever solution, washed and counted as described previously (4). Rats were immunized intravenously via the lateral tail vein by a single dose of 4×10^8 erythrocytes (4).

Measurement of antibody-forming cells (AFC) of spleens. The number of splenic cells capable of synthesizing hemolytic antibodies was determined by the method of Jerne *et al.* (11) with the modifications previously described (4). Briefly, this method involves removal of spleens from immunized rats and incubation of a suspension of splenic cells with sheep erythrocytes in agar. Addition of fresh complement subsequently causes lysis of the sensitized erythrocytes in the immediate vicinity of individual AFC. The clear plaques surrounding each AFC are counted.

Results. The effects of the various deficiency states on the number of splenic AFC in immunized rats are summarized in Table I. Since the number of AFC reaches a maximum value on the fourth day after immunization with sheep erythrocytes (4), AFC were determined only 4 days after immunization in the present study. It is apparent that the number of splenic AFC was reduced in all of

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TABLE I. ANTIBODY-FORMING CELLS (AFC) IN THE SPLEENS OF THIAMIN, RIBOFLAVIN, BIOTIN AND FOLIC ACID-DEFICIENT IMMUNIZED RATS.[a]

Group	No. of rats	AFC/10^6 Splenic cells		Body weight (g)[b]	
		Average	Range	Initial	Final
Control	8	160	146–200	56 ± 1	242 ± 9
Thiamin-deficient	7	59	39–81	64 ± 5	76 ± 6
Riboflavin-deficient	6	16	5–42	57 ± 6	105 ± 23
Biotin-deficient	6	4	1–6	60 ± 5	130 ± 18
Biotin-deficient, treated[c]	5	54	49–63	60 ± 5	166 ± 16
Folic acid-deficient	8	10	2–54	62 ± 8	177 ± 32
Folic acid-deficient, treated[d]	5	34	6–62	62 ± 8	185 ± 38

[a] Sheep erythrocytes, 4×10^8 per rat, were administered intravenously 4 days before removal of the spleens.
[b] Body weights are expressed as average ± SE. Final body weight was recorded at the time of sacrifice.
[c] Each rat received a daily intraperitoneal injection of 10 μg of biotin beginning 3 days before immunization and continued until the end of the experiment.
[d] Each rat received a daily intraperitoneal injection of 50 μg of folic acid beginning 3 days before immunization and continued until the end of the experiment.

the deficiency states under study, with the least effect in thiamin deficiency. Daily administration of biotin or folic acid for 7 days beginning 3 days prior to immunization partially restored the ability of the corresponding deficient animals to produce AFC.

Discussion. The present study has demonstrated the varying effects of thiamin, riboflavin, biotin and folic acid deficiencies upon AFC formation. We have previously reported that the production of circulating antibodies following the administration of diphtheria toxoid was also reduced to variable degrees in these deficiency states (9). Of particular note is the observation that thiamin deficiency which had the least effect upon formation of AFC also had the least effect upon the production of circulating antibodies. Direct comparisons of the effects of these deficiencies upon circulating antibody and AFC formation are difficult since both the antigens and the mode of assessment of the immune responses differed in the two studies. Whereas the cellular response reported in the present paper is associated with IgM formation (12, 13), the serum antibody titers determined 3–4 weeks after immunization with diphtheria toxoid (9) reflects both IgM and IgG concentrations (14).

The adverse effects of the deficiency states of thiamin, riboflavin and biotin may not be attributed to the delayed formation of AFC after immunization since the number of splenic AFC in the deficient rats was less on day 5 than on day 4 after immunization. Thus, the number of antibody forming cells 5 days after immunization in thiamin, riboflavin, and biotin-deficient rats was 47, 8 and 1, respectively. Previous studies with inanition controls have demonstrated conclusively that inanition does not have a deleterious effect upon the formation of AFC (4, 5). A similar lack of effect of inanition *per se* upon immune responses has been noted frequently (1). The effects of the deficiency states upon the formation of AFC in the present study can, therefore, not be attributed to inanition.

In contrast to the failure to restore completely the capacity to fabricate AFC with short-term therapy with either biotin or folic acid, this decreased cellular immune response was restored to normal by similar administration of vitamin B_6 or pantothenic acid to deficient animals (4, 5).

The precise function of the B-complex vitamins in the development of AFC is not known although the adverse effects of vitamin B_6 deficiency have been linked to the role of pyridoxal phosphate in the formation from serine of one-carbon unit required for the synthesis of DNA and RNA involved in antibody formation (15, 16). The immune response is a complex process involving the discrete steps of antigen processing, differentiation of progenitor cells, replication of plasma cells, antibody synthesis and transport of newly-synthesized antibodies into the circulation. Much further study will be required to delineate those steps whose rates are controlled by the metabolic activities of the coenzymatic forms of these vitamins. Current investigations in this laboratory involve studies

effects of these deficiency states upon antitation of various cell types involved ibody synthesis and their interactions.

ımary. The formation of splenic anti-forming cells was determined by the agar-plaque technique in normal rats ı rats deficient in thiamin, riboflavin, or folic acid immunized with sheep ocytes. The number of antibody-form-lls was reduced in all deficiency states hiamin deficiency having the least ef-his decreased cellular immune response :in and folic acid deficiencies was par-estored to normal by administration of or folic acid, respectively, shortly be-ımunization.

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Increased Lethality After Endotoxin in Old or Leukemic AKR Mice[1, 2] (40069)

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In the course of studies in which endotoxin (LPS) was given prior to whole body irradiation (WBI) of mice with AKR lymphoma in hopes of obtaining greater damage to the lymphoma (1–3) while promoting earlier regeneration of the normal hematopoietic system (4, 5), it was observed that relatively small doses of LPS killed the older lymphoma bearing AKR mice but not the young controls. Consequently, the present series of studies was carried out to begin to determine the nature of this difference in sensitivity to LPS. Since AKR mice do not develop spontaneous lymphoma until after they reach about 6 months of age the observed sensitivity to LPS could have been due either to the presence of the lymphoma or to an effect of having reached the age at which lymphoma develops. In order to determine which of these factors were involved, comparisons were made of LPS sensitivities of "old" (6–8 months) mice without evidence of lymphoma, "old" mice with spontaneous lymphoma, "young" (3–4 months) mice with transplanted lymphoma and "young" nonlymphoma bearing mice. After these different sensitivities were defined efforts were made to determine whether or not some factors which are known to change with or be associated with responses to LPS might be different in these mice.

Materials and methods. Mice were AKR "young" adult (3–4 months) or "old" (6–8 months) retired breeder or virgin females from either Jackson Laboratories, Bar Harbor, ME, or Charles River Breeding Laboratories, Wilmington, MN, housed 5–10 per cage at about 23–25°C with food and HCl (pH 2.4) water *ad libitum*. During the experiments mice were checked at least once daily and dead animals removed for necropsy. Mice with lymphoma have grossly enlarged spleen, thymus and inguinal lymph nodes at necropsy. The median and ranges of body weights were 27 (23–31) g for 6 month old mice used and 24 (20–28) g for 12-week old mice. Although 6–8 months would be considered to be middle age for many strains, AKR mice have reached 70–90% of their mean life span at 6–8 months and begin to die of spontaneous thymic leukemia, and were therefore defined as "old".

The endotoxin used was *Salmonella typhosa* lipopolysaccharide B 0901 (LPS) from Difco, Detroit, MI. The lyophilized material was diluted to a concentration of 2000 μg/ml in 0.9% NaCl, and stored frozen at −10°C in 25 ml aliquots. The same 2-year old stock solution was used for all of the studies, and was diluted the day before intraperitoneal injection. Aliquots were given as μg LPS per mouse. Death after injection of LPS usually occurred within 1–3 days and data are expressed as 3-day mortality.

The radiation source was a Picker X-ray Unit operating at 200 KV, 15 mA, with 0.25 mm Cu and 1 mm Al filtration. The dose rate of 100 rads/min was calculated from the dose measured by Victoreen dosimetry in air at the center of the plastic mouse container. Dry weights of intestines were obtained as previously described (6).

The transplanted lymphoma was from the 24th and 92nd passage of 10^6 spleen cells collected just prior to death of previously injected mice. Death after each passage occurred within a very tight time span after injection (11 ± 2 days and 7 ± 2 days for the passages used). Endotoxin was injected 5–6 days before the time of expected death.

Reticuloendothelial (RE) function was tested by measuring rates of clearance of carbon from the blood. Colloidal carbon was Gunther Wagner's (Special Ink) #C11/1431A (generously given to us by Dr. Lola Kelly) and prepared according to the

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[2] We appreciate the technical assistance of Kenny Patrene and Marilyn Walter.

[3] Leukemia Society of America Scholar.

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scribed by Halpern *et al.* (7) and Finney (8). Blood was obtained e of the retro-orbital sinus with a l capillary tube. For platelet of blood was diluted in 100λ of n into a segment of Teflon tubing sealed at one end, spun at 150 g and platelet rich plasma was di- Isoton for electronic counting. e expressed as platelets per mm^3 d. Nucleated cell counts were also y Coulter Counter and differen- ade from 100 Wright stained cells p blood smears.

used include, in the case of gut lculation of mean ± 1 SE and, in blood cell counts calculation of nean ± 1 SE. For survival studies, were compared by chi-square in tests or Fisher exact confidence s where each was appropriate (9). geometric means were compared tudent's *t* test. Standard errors of ad were calculated by $\sqrt{\frac{pxq}{n}}$ percent dead, q = percent alive mber of animals tested. A proba- ss than $P \leqq 0.05$ was taken to gnificant differences. LD_{50} data ed by probit analysis.

Effect of age. When survival was fter various doses of LPS given to 212 "old" and apparently non- ice and 261 "young" mice, it was t the lower doses the "old" mice more sensitive to LPS than the ice. Data for "old" virgin females breeder females from Charles ckson Labs were not significantly nd data were pooled. A single in- LPS was not lethal in young adult ses of 64 μg or less (Fig. 1). In thal effects were seen at doses as g in "old" mice. Both curves for old mice tended to level off at doses, where 256 μg killed only e "young" mice and 94% of the . Probit analysis gave LD50/3 val- 50 μg for "young" mice and 28 "old" mice.

f lymphoma in "young" mice. If njected 5–6 days prior to the ex- e of death for "young" adult mice with transplanted lymphoma (total of 70 mice) the lethal effects were much greater than in the normal nontransplanted controls. Deaths in the mice with lymphoma were seen with a dose as low as 25 μg LPS and 100% were killed by 100 μg compared to 53% in the normal controls (Fig. 2). Unlike the control curve, the curve for lymphoma mice failed to level off at high LPS doses.

Effect of lymphoma in old mice. Since at 6 months of age some AKR mice begin to show clinical signs of lymphoma and the transplanted lymphoma caused increased sensitiv-

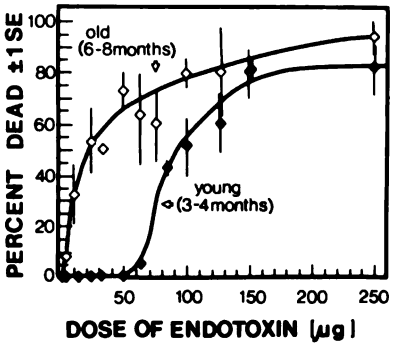

FIG. 1. Percent deaths in young (3–4 months) and old (6–8 months) AKR mice given 1, 5, 10, 16, 25, 32, 50, 64, 75, 100, 128, 150 or 256 μg *Salmonella typhosa* endotoxin. Deaths were cumulative over 3 days after ip injection of the endotoxin into mice. Old mice (open diamonds) were either virgin or retired breeder females. Young mice (closed diamonds) were virgin females. The variations shown were calculated as $\sqrt{pq/n}$. The two curves were significantly different at $P < 0.001$.

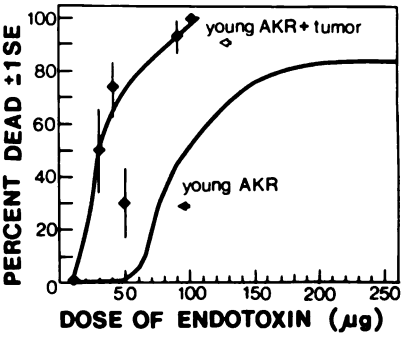

FIG. 2. Percent deaths in young mice without (from Figure 1) and with a growing transplanted AKR lymphoma (closed diamonds) 3 days after various doses of endotoxin. None of the tumor bearing mice died within the 3-day interval if no endotoxin was injected. These curves were different when paired values are compared ($P < 0.001$).

ity to LPS, the status of the "old" mice with regard to lymphoma was determined. Mice with advanced lymphoma were identified by their obviously enlarged lymph nodes and spleen and excluded from our study as they often die just from the trauma of handling. The 74 "old" mice with lymphoma included in the study had either no palpable lymph nodes or spleen, or nodes that were palpable but not grossly enlarged. At death, or at termination of the experiment all the mice were necropsied and classified as either having lymphoma or not. The "old" mice with spontaneous lymphoma showed no evidence of greater sensitivity as compared to "old" mice without lymphoma (Fig. 3). However, the shape of the curves for "old" mice with lymphoma, like that of mice with transplanted lymphoma may have been different from that of mice with no lymphoma in that they failed to level off at high doses of LPS.

Effect of preirradiation. Smith *et al.* (10) have shown that radiation increased sensitivity to LPS injection. This increased sensitivity was greatest 3 days after radiation and was prevented by shielding the gut but not by shielding the marrow from radiation. We tested the combined lethal effects of radiation and endotoxin on "old" and "young" AKR mice (Table I). LPS was injected 3 days after radiation and deaths were recorded for the next 3 days (to 6 days postirradiation). The data provide some evidence that "old" mice were more sensitive to radiation since higher death rates were recorded for "old" than for "young" mice at all radiation doses even when no LPS was given. It is also evident from these data that the combined effects of radiation and LPS are more lethal in "old" than in "young" AKR mice.

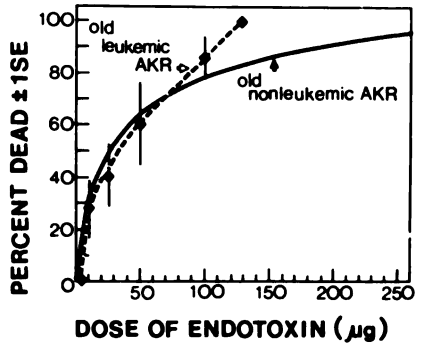

FIG. 3. Percent deaths in old nonleukemic mice (from Fig. 1) and old leukemic mice (closed diamonds) given various doses of endotoxin. These curves were not significantly different ($P \cong 0.5$).

In old mice all doses of radiation resulted in greater sensitivity to endotoxin significant at $P \simeq 0.05$ for 400 rads, $P \simeq 0.005$ for 500 rads, and $P < 0.001$ for both 600 and 700 rads (data in Table I). In young mice only the highest dose (700 rads) resulted in significantly increased sensitivity to endotoxin ($P < 0.001$) (see Table I); 400, 500 or 600 rads failed to do so. No deaths were seen in any of these mice at doses of endotoxin below 32 μg. At 32 μg, 64 μg and 128 μg the combined data for 400 to 600 rads gave 1/40 (3%), 6/30 (20%) and 14/25 (56%) deaths respectively.

Tests for possible causes of different sensitivities. Since previous data suggested that the greater sensitivity to endotoxin seen after radiation might be prevented by gut shielding (10) and since other data have shown that dry weight of the small intestine reflects radiation damage to the mucosal tissue (6), we measured the change in small intestine dry

TABLE I. DEATH OF "YOUNG" AND "OLD" AKR MICE EXPOSED TO RADIATION AND GIVEN *S. typhosa* ENDOTOXIN 3 DAYS LATER.

Endotoxin dose (μg)	Number Dead/Total Tested (%)[a]				Young (3–4 months)
	Old (6–8 months)				
	400 rads	500 rads	600 rads	700 rads	700 rads
0	0/12 (0)	0/13 (0)	4/30 (13)	10/20 (50)	1/15 (7)
1–2	1/6 (17)	2/27 (7)	6/28 (21)	8/23 (35)	4/20 (20)
4–5	2/6 (33)	5/12 (42)	5/13 (38)		0/20 (0)
8	0/6 (0)	2/13 (15)	3/14 (21)		0/10 (0)
16	3/13 (23)	10/14 (71)	9/13 (69)		8/15 (53)
32	9/13 (69)	5/6 (83)	14/14 (100)	14/14 (100)	9/15 (67)
64	12/12 (100)	4/5 (80)	29/29 (100)	13/13 (100)	
128	6/18 (33)	14/14 (100)	29/29 (100)	14/14 (100)	

[a] Nonirradiated control data from these experiments were included in the figures.

on days 2, 3 and 5 after 700 rads on. There was no difference in gut between the "young" and "old" non- c mice. Weights dropped to 82 and pectively on day 2 and returned to ormal levels on day 1.

urement of gut weights of "old" leu- ice was complicated both by loss of e to their greater radiosensitivity and presence of enlarged Peyer's patches ecome small after irradiation. When tches were removed before weighing, ern of gut weight change began the opping to 82% on day 2) but no mice day 5 and all the weights were lower nonleukemic mice.

nction was tested by determining the emoval of colloidal carbon from the fter iv injection. The half times for clearance were measured in "young" na bearing mice and non-lymphoma "old" mice. Both were able to remove carbon from their blood as rapidly young" controls tested on the same $t_{\frac{1}{2}}$ of 34 ± 2 minutes for control and or lymphoma and 30 ± 4 for control ± 1 for "old" groups).

neutrophils and platelets can partic- the removal of injected LPS (11–17) nulocytes have been said to partici- activation of intravascular coagula- l precipitation of soluble fibrin by in (18). Blood cell counts in "young" l" mice and mice with transplanted na done 3 days posttransplantation significant differences in both the cyte and platelet counts. Mice with nted lymphoma had higher blood cyte counts (5300 ± 610/cubic mm) ver platelet counts (1.2 ± 0.24 × c mm) than "young" control mice 200/cubic mm and 3.6 ± 0.24 × c mm). Similarly "old" mice had at higher granulocyte counts (3000 ubic mm) and much lower platelet 0.86 ± 0.14 × 10^6/cubic mm) than ng" controls.

ssion. The present results indicate h "old" age and presence of lym- an cause greatly increased sensitivity oxin (LPS). The largest differences in ty between "young" and "old" mice n at lower LPS doses. At higher LPS th groups had some resistant mice. The presence of lymphoma seemed to affect the mice given high as well as low LPS doses. At the lower doses of LPS the presence of spontaneous lymphoma, however, did not increase the sensitivity over that of "old" mice. It seemed possible from this that the "old" mice might have reached a maximum sensitivity. These data may indicate that differences in sensitivity associated with age difference is different than that associated with lymphoma.

There are a number of circumstances which lead to greatly increased sensitivity to LPS. These include, adrenalectomy (19), altered reticuloendothelial function (20–22) or blockade (23), vaccination with BCG (24), serial zymosan injections (25), elevated ambient temperature (26–27), previous irradiation (10), irradiation plus acute graft versus host reaction (17), and immunosuppression (28). When we used one of these, irradiation, it was possible to further increase the sensitivity of the "old" mice. The relative increase in sensitivity due to irradiation, though evident in lower radiation doses in "old" than in "young" mice, was hard to evaluate because the "old" mice proved also to be more sensitive to the radiation.

Many previous studies have shown that "old" mice are more sensitive to radiation than "young" adult mice (29–33) but the strains of mice used in those studies are not "old" or more radiosensitive until they reach about 17–20 months, and in our studies the "old" mice were only 6–8 months old. In terms of life span, however, these ages are more alike. Thus the responses to LPS and radiation of "old" AKR mice may reflect early senescence.

Mechanisms which could be responsible for increased sensitivity to exogenous LPS include increased release of endogenous LPS from the gut (9, 15, 17, 37–45); decreased ability to remove or detoxify LPS (11–17, 20–23, 45–47); increased responsiveness of physiologic mechanism(s) leading to death; and loss of "protective" homeostatic mechanism(s).

There is evidence that the changes in the gut may be involved in these sensitivities. Gut radiosensitivity increases steadily with age from 3 months to 23 months in C57Bl/6J mice (32, 33). Studies of Smith *et al.* (10) showed that radiation-induced increased sen-

sitivity to LPS could be prevented by GI shielding, and other data suggesting changes in mucosal transit time in the gut with aging (34). These, taken together, prompted us to measure postirradiation small intestinal weight changes. In the present studies gut weights decreased to the same degree and increased at the same time and rate in both "old" and "young" mice. Gut weight is a crude measure of mucosal changes after irradiation although it has been useful in other studies (35, 36) in reflecting mucosal damage and regeneration. It is still possible that the vascular or mucosal integrity might be damaged by radiation or injection of LPS and this might lead to more release of endogenous LPS from the gut of "old" or leukemic AKR mice.

In our studies the capacity for RE clearance was studied by determining the $T_{1/2}$ for the removal of colloidal carbon from the blood. The half time for carbon clearance in young lymphoma bearing mice and nonleukemic mice was not significantly different from that of young normal control AKR mice. These studies suggest that RE clearance was normal and that a decreased ability to remove LPS by the RE system is not the mechanism for the observed sensitivity differences.

Blood neutrophil concentration was higher but platelet concentration was lower in "old" mice or young tumor bearing mice as compared to "young" mice. Endotoxin has an affinity for membranes (48) and injected endotoxin rapidly becomes associated with cells of the buffy coat, particularly platelets (11–16). Although the significance of these interactions has not been established, animals which survive LD_{50} doses of endotoxin are able to maintain adequate levels of granulocytes, platelets and coagulation factors, but those that die do not (49). Both granulocytes and platelets appear to have some ability to reduce mortality caused by endotoxin (11–17, 37) but, conversely, both cell types have also been implicated in the development of the pathophysiologic aspects of endotoxemia (18). For example, there is the possibility that elevated granulocyte counts at the time of LPS injection may cause more injury by release of more lysosomal constituents. It is clear that in the progression toward final cardiovascular collapse these cells participate, but it is not clear how the process starts or why it might be worse in "old" or "leukemic" mice.

Summary. Lethality following a single injection of *Salmonella typhosa* endotoxin (LPS) was found to be higher for 6- to 10-month "old" AKR than for "young" adult (3 months old) of the same strain. "Young" AKR mice with transplanted lymphoma were also more sensitive to the lethal effects of endotoxin than the nonlymphomatous "young" mice. Exposure to radiation 3 days prior to endotoxin injection increased sensitivity in both the normal "young" mice and the "old" AKR mice but significant increases were seen at lower radiation doses in the "old" mice. "Old" mice were more sensitive to the radiation than "young" mice. Several possible causes for differences observed were investigated. Reticuloendothelial function as measured by rate of clearance of colloidal carbon from blood was the same for "young" and "old" mice. Previous studies have implicated the gut in the increased sensitivity to endotoxin seen 3 days after radiation. The present studies showed no differences between young and old in degree of small intestine weight loss or rate of recovery after 700 rads X-ray exposure. Although counts of blood granulocytes were somewhat higher and platelets were somewhat lower for the "young" tumor-bearing and "old" mice as compared to "young" controls, it is not obvious how this might affect the differences in LPS sensitivity observed. These data suggest that effects of "old" age, lymphoma, or combined "old" age and radiation may lead to unexpected lethal effects from relatively small doses of endotoxin. The biological reason for this increased sensitivity remains obscure.

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Regulation of Food Intake During Long-Term Loss of Food from the Intestines of the Rat (40070)

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It has long been established that rats adjust to caloric dilution of their diet by increasing the amount of food eaten. The adjustments have occurred within 1 day when the food was diluted prior to feeding (1, 2) or before injection into the stomach (3, 4). In the present study, rats that lost a substantial portion of their ingested food from the intestines failed to increase their food intake for 5 days. During this period, the amount of food eaten appeared to be regulated by signals that arose in the mouth, throat, stomach, proximal duodenum or the lower intestines.

Surgical preparation. Eight pairs of parabiotic Lewis rats were prepared by uniting the skin and muscle of the abdomen (5). A circular opening with a diameter of 3–4 cm was made in the abdominal wall of each rat of a parabiotic pair to produce a common peritoneal cavity. In four pairs of parabiotic rats, a 30 cm segment of one rat's intestines was exchanged for an equivalent segment of its partner's intestines. This was accomplished by transecting the intestines of each rat about 5 cm below the stomach at midduodenum. The proximal ends of each rat's duodenum were united by 7–0 Connell sutures to the distal ends of its partner's duodenum (see Fig. 1). The intestines were crossed back 30 cm further down the digestive tract at midjejunum. In this surgical preparation the food eaten by one rat arrived in its own stomach, traveled through 5 cm of its own proximal duodenum to the distal duodenum of the other rat. It then traversed 30 cm of the other rat's distal duodenum and proximal jejunum before the remaining unabsorbed food returned to the intestine of the rat that fed. The crossed segment is more than one quarter of the total length of the rat's small intestines (about 110 cm) and is located in the most proximal region. The rats recovered from surgery after a few days and continued to gain weight in the postsurgical period. They were normally vigorous and showed no signs of stress once an adjustment to paired life had been made.

Absorption study. The food eaten by one rat of a crossed intestines pair was absorbed from the intestines into the blood of both rats. The percentage of food absorbed into each rat can be estimated by measuring the relative amounts of a fed radioactive nutrient that has been absorbed into the blood of each rat. Three pairs of each type of rat were included in the study. The rats were deprived of food for 12 hr and then one rat of each pair was injected with a meal at the rat's average feeding rate (9 ml/8 min) through a chronically implanted stomach tube (6). The injected meal consisted of 9 ml of their normal liquid diet, Nutrament (Mead–Johnson Nutritionals) which contained 60% carbohydrate, 29% protein and 11% fat by weight. The liquid diet meal was thoroughly mixed with trace amounts of glucose-^{3}H or amino acid-^{3}H mix (International Chemical and Nuclear Corp.). One-tenth milliliter of blood was withdrawn from each rat's tail just before the injection and every 10 min after the injection began. The blood sample was precipitated in 3 ml of Bray's solution and 1 ml of the fluid was withdrawn, diluted with 20 ml of Bray's solution and counted in a Beckman 250 scintillation counter. Counting efficiency was essentially constant for all of the sample tubes.

Results. As can be seen in Fig. 2, the glucose-^{3}H was rapidly absorbed into the bloodstream of the fed parabiotic rat. Only a small quantity of radioactive label was transferred through the capillaries in the skin and muscle of the parabiotic union to the bloodstream of the unfed parabiotic rat. In contrast, glucose-^{3}H mixed into the diet fed to one of the crossed intestines rats was rapidly absorbed into both rats. There was no significant difference in the blood levels of radioactive glucose in these rats at 10, 30, or 60 min ($F = 0.29$ at 60 min, $P > 0.25$).

Figure 3 presents the same data when

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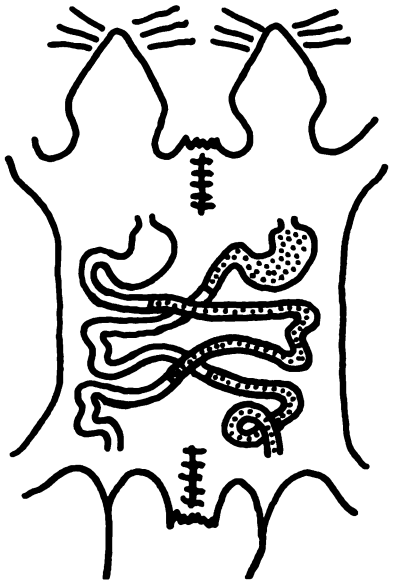

. 1. Diagram of a crossed intestines rat pair. The
ɔd gastrointestinal tract belongs to one rat and has
ɔd supply and neural connections intact.

ɔ acid-^{3}H mix (a typical algal protein
ɔlysate with 15 common amino acids)
ɛen added to the diet which was injected
ɔne rat of each pair. Again, approxi-
y half of the radioactive amino acids
absorbed into the bloodstream of each
ʌt ten minutes, a significantly greater
nt of amino acid had been absorbed
he unfed crossed intestines rat than into
artner rat that was fed ($F = 18.3$, $P <$
or into the parabiotic control rat that
ɛd ($F = 29.1$, $P < 0.01$). Since approxi-
y equal amounts of radioactive amino
were present in the fed rats of each pair
0.07, $P > 0.25$), the data suggest that
was initially a more rapid emptying of
ɔmach contents of the fed crossed intes-
ɾats. These stomach contents passed into
ɹrtner rat's intestines and were absorbed
during the next 10–20 min. By 30 and
n, the blood levels of the amino acids in
crossed intestines partner were not sig-
ntly different ($F = 0.03$ at 60 min, $P >$
suggesting that the initial rapid rate of
ɹch emptying had been inhibited by nor-
hysiological processes in the fed crossed
ines rat. Once the initial adjustments
a meal were complete, the radioactive
ɔ acid was divided about equally in the
ɾossed intestines rats.

Both absorption studies show that approximately half of the free glucose or amino acids present in the food eaten by one crossed intestines rat was absorbed into each rat of the pair. These measurements provide only a rough estimate of the amount of food absorbed into each rat. First, the diet contained complex foods that required enzyme digestion and would not necessarily be absorbed

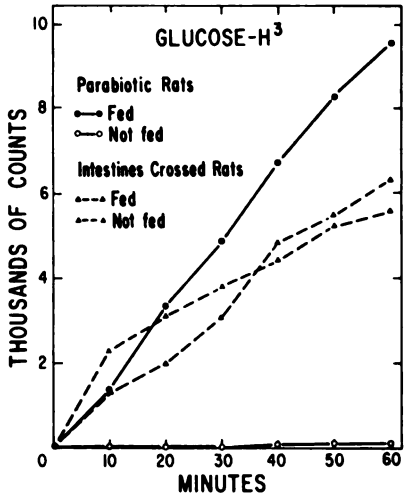

FIG. 2. The number of radioactive counts present in a 0.1 ml sample of blood taken every ten minutes from each rat's tail after one rat in each pair had been infused with its normal diet mixed with trace amounts of glucose-^{3}H. Data is presented for both the fed (solid symbols) and the unfed (open symbols) rats of each type of pair.

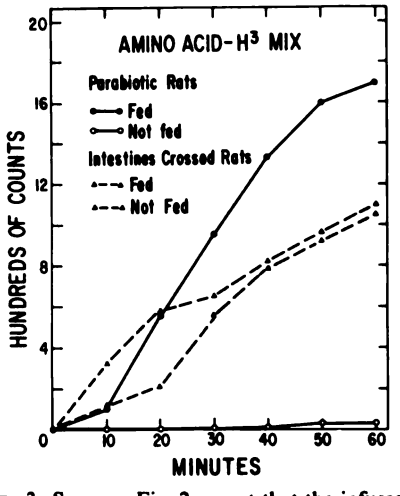

FIG. 3. Same as Fig. 2 except that the infused meal has been mixed with tritium labeled amino acid mix.

at the same rate as free nutrients. Second, the blood levels of radioactive nutrients are influenced by two dynamic processes: their absorption from the gut and their removal from the blood by the body's tissues. If rate of removal differed in the two rats, then the blood levels of the radioactive nutrients would not reflect the absorption rate. Fortunately, the blood levels of the labelled nutrients are approximately the same in the crossed intestines rats, and thus, rate of removal is not likely to be greatly different.

Behavioral study. Both absorption studies show that a substantial proportion of the food eaten by one crossed intestines rat was absorbed into each rat of the pair. Under normal circumstances when both rats were feeding, each rat absorbed the amount of food that it ate. The food lost from the intestines of one rat was gained from the food eaten by its partner. Only when the food intake of one rat was restricted, as in the following behavioral study, did the feeding rat suffer a loss of nutrients.

In the behavioral experiment, the rats were placed on a twelve hour food deprivation schedule. They were allowed to eat during the night from 9:00 PM until 9:00 AM while kept restrained in modified Bollman restraining cages (7). These cages had a plastic partition that prevented the rats from eating each other's food. During the daytime hours, the rats were returned to home cages where they could exercise. They had water freely available at all times.

The results of the experiment are presented in Fig. 4. During the first 5 days, the amount of food eaten by each rat stabilized and the weight of the rat pairs remained constant. On the 6th day the liquid diet, Nutrament, fed to the rats was diluted with an equal quantity of water. As in previous studies (1, 3) the rats increased the volume of food eaten within one day, but they never fully compensated for the caloric dilution. They ate on the average 1.66 times their normal food volume and consequently lost some weight during the three days of food dilution. During days 9–11, the rats were again fed their normal diet and their weight stabilized.

On the 12th day, the critical experiment began. Only one rat of each pair was allowed to feed. For the parabiotic rat pairs, no adjustment in food intake was expected. The parabiotic rat that was allowed to eat would feed itself. A small amount of nutrient in the blood would pass from this rat through the capillary connections in the parabiotic union to the partner rat. However, all blood returning from the partner rat would contain mobilized free fatty acids, amino acids and glucose. Very little net loss of nutrient from the feeding parabiotic partner would be expected. As can be seen in Fig. 4, the fed parabiotic rats did not increase their food intake throughout the five day period as had been expected. ($F = 0.06$, $P \gg 0.25$).

On the other hand, the fed crossed intestines rat would lose a substantial portion of its food to the intestines of its partner (see Figs. 2 and 3). However, no food would enter its own crossed intestines segment because its

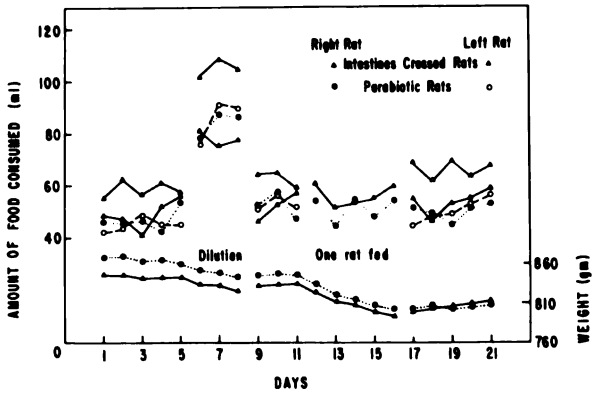

FIG. 4. The daily food intake and body weight for pairs of crossed intestines rats and parabiotic rats. The food intake for the left and right rats in each pair were plotted separately. The left rats of both pair types were not fed on days 12–16.

er was not fed. If the feeding behavior
: fed crossed intestines rats were affected
ıysiological signals normally arising in
ʏn crossed intestinal segment or by the
amount of food absorbed, the fed rat
d have increased the amount of food
to compensate for the food lost to its
er. As can be seen in Fig. 4, the fed
ɔd intestines rats did not increase their
intake for 5 days. The amount eaten
g these days is not significantly different
the amount eaten during the preceding
ubsequent control periods ($F = 0.86$, p
25). The failure of these rats to increase
intake when a substantial portion of the
ɛnt was lost from the intestines to the
er did not result from the lack of stom-
apacity. When the diet was diluted by
ɥual volume of water, all the rats in-
ɔd the amount eaten by an average of
nl, an increase that is statistically highly
ïcant ($F = 175.5$, $P < 0.001$). The stom-
of these rats must have held the in-
ɔd volume of the diluted diet until the
ɨ water had been slowly absorbed.
over, the average largest intake on a
: day for each of the experimental rats
g the control period is 7.3 ml more than
verage intake during the intestinal dilu-
The rats could have adjusted to loss of
ɛnt by consistently eating at least an
ional 7.3 ml of liquid diet each day, but
did not do so. On days 17–21, food was
to both rats of each type of pair. Food
e of both the fed and previously unfed
vas normal during this period, and the
lowly gained weight.

ɟure 4 shows that the rats ate their usual
tity of food when only one rat of each
was allowed to feed despite the loss of
t 50 g of body weight for both types of
air. This loss was approximately 6% of
body weight and comprises about one
of the body fat of normal rats (8). If we
ne that the glucose and amino acid ab-
ion curves give a reasonably fair esti-
of the amount of food absorbed, then
t one third of the body fat was lost in
member of the crossed intestines pairs
bout two-thirds of the body fat was lost
: unfed parabiotic rats. This assumption
not tested by carcass analysis in this
because measurements of postdepriva-
tion food intake and return of body weight were desired. Carcass analysis would provide a check on the assumptions about the amount of food absorbed in each crossed intestines rat and has been incorporated in current experiments. In the present study, the deprivation period was restricted to 5 days in order to maintin the unfed parabiotic rats in a healthy condition since rats die after 7–8 days without food (9).

Discussion. Two previous studies have crossed the intestines of rats without crossing them back. In one study, the intestines were crossed just below the stomach (10) and in the other, at midduodenum (11). In both studies, the feeding behavior of the few surviving rats was highly erratic with one rat eating continually while its partner stopped eating at all. This peculiar pattern of eating behavior which I have also observed on similar preparations was probably due to the loss of an important reflex that affects intestinal motility. The gastroileal reflex mediated by the release of gastrin after food enters the stomach, causes food to move from the small to the large intestines (12). When the intestines is crossed in only one direction, the gastrin is released in one rat but the target organ, the terminal ileum, is in the other rat. Thus, feeding does not lead to normal movement of food along the intestines. I have done one short-term food intake experiment on rat pairs which have the intestines crossed over and back (6). In this previous study, the crossing of the intestinal segment occurred 10 cm further down the intestinal tract at mid-jejunum and the crossed segment was only 25 cm long. A glucose-^{3}H absorption study similar to the one reported here has shown that most of the radioactive glucose was absorbed into the fed rat of those crossed-intestines pairs. Since free gluose is absorbed in the upper portions of the digestive tract (13), the glucose-^{3}H absorption study in the previous paper gave an underestimate of the amount of food absorbed. This conclusion was supported by the concurrent measurement of the blood levels of the less rapidly absorbed sugar, 3-O-methyl-*d*-glucose[^{14}C], in a double label experiment. A comparison of the absorption curves of both studies shows that the additional 10 cm of lower duodenum and upper jejunum included in the crossed

segment of the present study is important for the absorption of free glucose. This crossed segment is also an important site for the absorption of complex carbohydrate, protein and fat (12). The present study extends the previous results by crossing a longer and more proximal segment of the small intestines and by extending the test feeding period from one hour to 5 days. The results of both studies are consistent: crossing the intestines of rat pairs does not alter food intake during the first hour of feeding or during a 5-day period.

In all studies, rats have adjusted to dilution of their diet by substantially increasing their food intake. Even rats that have been trained to press a lever to deliver food directly to their stomach were able to compensate for changes in their diet (3, 4). When their diet was diluted with an equal volume of water, they doubled the volume of diet delivered to their stomach. When each lever press delivered a smaller amount of food, they increased their lever pressing to compensate for the lost nutrient. In the present study, the rats need only increase the amount of food eaten to compensate for nutrient loss from its intestines. Despite the loss of a substantial portion of the fed nutrient, the rats did not increase their food intake during a 5-day period. Apparently, the rats will compensate for a decrease in food delivered to their stomachs but not for food lost from their intestines. A satiety signal must arise from those sections of the digestive tract that are stimulated by food. In the feeding rat, these sections include the mouth, throat, stomach, proximal duodenum and the lower jejunum and ileum. The signal is either neural or hormonal since it appears to be affected by the amount of food present in the stomach and some sections of the small intestines, but not by the amount of nutrient absorbed. This internal signal continues to control food intake for 5 days even though a substantial amount of the food eaten is lost from the intestines, the metabolism of the rat shifts to a greater dependence on free fatty acids and body weight is reduced by 6%.

Summary. Four pairs of parabiotic rats had a thirty cm segment of each rat's distal duodenum and proximal jejunum exchanged for the same segment of the partner's intestines. The food eaten by one rat of each pair was absorbed in nearly equal amounts into both rats. When the liquid diet was diluted with an equal volume of water, the rats adjusted by nearly doubling their food intake in 1 day. When the rats lost a substantial portion of their ingested food from their intestines, they failed to increase their food intake during 5 days. The amount of food eaten was unaffected by a considerable decrease in the amount of absorbed nutrient and a 6% decrease in body weight.

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agen Composition of the Skin from Obese and Lean Zucker Rats (40071)

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netically obese Zucker rat transmits s a Mendelian recessive trait (1). nt is frequently studied as a model e-onset obesity in humans because e Zucker rats (2) and certain types humans (3) demonstrate many simbolic, endocrinologic and morphoormalities. Although major differipid metabolism between obese and als and humans have been reported, lterations in protein metabolism in e much less well defined.

ant deviations in growth between l lean Zucker rats occur at an early at continues to accumulate progresoughout life (4, 5). Carcass analyses hese observations; obese rats demignificant increases in body fat and in carcass protein and water comtheir lean littermates, even under ng conditions (6, 7). Obese rats are t in their utilization of dietary nitro- compared to lean rats; less nitrogen ed to body protein and more nitrocreted by the obese (8, 9). Physical ncreases the body protein content of Zucker rat but not to the level of 10). An impairment in growth horduction has been suggested as an on for the generation of stunting ity in the obese Zucker rat (4). normone deficiency in children rewth and leads to increased deposidy fat; growth hormone administrases these effects (11). Obese humans ate a significantly blunted response i which increase growth hormone lean subjects, e.g., exercise (12), ar-, 14) and l-dopa (15, 16).

l protein metabolism in obesity is ested in studies of protein synthesis io acid levels. Obese Zucker rats ate increased levels of hepatic proesis (17, 18), however, the protein f livers from obese rats appears to be reduced compared to hepatic protein levels in lean rats (18). Circulating levels of leucine, isoleucine, valine, phenylalanine and tyrosine are elevated and glycine is reduced in obese compared to lean subjects (19).

The present study was designed to investigate collagen metabolism in skin of lean and obese Zucker rats. This protein and tissue were selected because of (a) the ubiquitous and abundant occurrence of collagen, and (b) the observation that the skin of obese rats tears and wounds more easily than the skin of lean animals (20). The results reported here indicate a normal collagen composition but an abnormal accumulation of lipid in the skin of obese Zucker rats. The reduced break strength of skin from obese rats, due probably to the decreased amount of collagen per cm^2 caused by a displacement of collagen fibers by lipid, may explain the frequent skin tearing.

Materials and Methods. Animals. Genetically obese rats and lean littermates purchased from L. M. Zucker (Harriet G. Bird Memorial Laboratory, Stow, MA) were housed in wire-bottomed cages in a temperature-regulated (22°) light-controlled room. Animals were fed Purina Laboratory Chow *ad libitum* prior to sacrifice.

Determination of skin collagen. The animals were killed by decapitation, their backs were shaved, a section (approximately 8 × 5 cm) of skin was removed from the midportion of the back and then the skin was freed of adhering subcutaneous tissues. Eight punches were made through the full thickness skin using a No. 8 cork borer. The skin punches were divided randomly into two equal samples, the wet weights of the samples were recorded, and then the samples of skin were dried by lyophilization. After recording the dry weight, the pieces of skin were extracted overnight at room temperature with 20 ml of chloroform–methanol (2:1). The pieces of skin were dried under a stream of nitrogen,

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and then the lipid-free dry weight was recorded. The skin samples were hydrolyzed at 105° for 24 hr in 5 ml of 6 *N* HCl. The amount of hydroxyproline in the hydrolysate was determined using a Technicon autoanalyzer (21). The hydroxyproline values were multiplied by 7.23 to obtain collagen equivalents (22).

Extraction and characterization of skin collagen. Skin samples (1–1.5 g) were minced and then shaken with 10 ml of 0.5 *M* acetic acid for 24 hr at 4°. Insoluble material was collected by high speed centrifugation, then reextracted. The supernatants were removed by decantation. The combined supernatants were dialyzed exhaustively against 0.05 *M* acetic acid, then dried by lyophilization. The skin residue after acid extraction was treated with pepsin according to the procedure of Chung and Miller (23). The pepsin-solubilized collagen was dialyzed at 4° against 0.15 *M* phosphate buffer (pH 7.6) and then against the same buffer containing 1.5 *M* NaCl. The precipitate which formed during dialysis was collected by centrifugation. The pellet was redissolved and the precipitation with 1.5 *M* NaCl repeated. The supernatants from the first and second precipitations were combined. The pellet was dissolved in 0.5 *M* acetic acid and then the dissolved pellet and the supernatant were dialyzed against 0.05 *M* acetic acid. Following dialysis, the samples were dried by lyophilization. Differential salt precipitation (23, 24) of pepsin-solubilized collagen yields a fraction enriched in Type III collagen (1.5 *M* NaCl-insoluble) and a fraction enriched in Type I collagen (1.5 *M* NaCl-soluble).

Break strength of skin. A section of back skin was removed and cleaned of hair, subcutaneous fat, and fascia. The full thickness skin was cut into strips 1.25 cm wide and the break strength of the skin measured using an Instron Universal Testing Instrument (Canton, MA) equipped with 100 kg tension load cell.

Results and discussion. Composition of the skin. Data concerning the chemical composition of skin from obese and lean Zucker rats are presented in Table I. The skins from the obese animals contained significantly more lipid per g wet weight than did the skins from their lean counterparts, even though all skins were free of visible subcutaneous fat and fascia. This effect which was more pronounced in females than in males was also more pronounced in the younger animals than in the older animals. The amount of water and of lipid-free dry residue per g wet weight was decreased in the obese skins when

TABLE I. COMPOSITION OF SKIN FROM OBESE AND LEAN ZUCKER RATS.

			Skin Composition[a]		
Animals	Age	Body weight	% H_2O	% Lipid	% Lipid-free dry-residue
Female lean	2 months (6)	170 ± 4	68.3 ± 1.6	8.2 ± 1.0	24.6 ± 0.4
	6 months (7)	—	59.8 ± 0.6	4.5 ± 0.3	35.6 ± 0.4
Female obese	2 months (6)	235 ± 8	41.2 ± 1.6[b]	41.9 ± 2.1[b]	16.8 ± 0.6[b]
	6 months (7)	—	42.5 ± 3.8[b]	36.1 ± 4.1[b]	21.4 ± 1.4[b]
Male lean	3 months (5)	256 ± 12	65.9 ± 2.0	6.7 ± 2.1	27.1 ± 0.7
	6 months (7)	—	61.0 ± 0.6	5.4 ± 0.4	33.5 ± 0.3
Male obese	3 months (4)	340 ± 12	46.5 ± 3.4[b]	34.4 ± 3.7[b]	19.1 ± 0.5[b]
	6 months (7)	—	53.9 ± 2.6[b]	18.5 ± 2.7[b]	27.6 ± 0.9[b]

[a] Percentage values for skin composition are expressed as percent of wet weight. The numbers in parentheses represent the number of animals in each group. Means ± SE of the mean are reported.

[b] These values for the skins from obese animals are significantly different from the values for the skins from age and sex matched lean animals at the level of $P < 0.05$ (Student's *t* test).

ɔd to lean skins. These data suggest re may be an atypical infiltration of o the skin of the obese Zucker rat.

ɟen content of skin. Table II shows the nparing the collagen content of skin ɔese Zucker rats with the collagen of skin from age and sex matched cker rats. When the collagen values ɹpressed as mg collagen per g wet ɔr per g dry weight, the skins from the ɪts contained significantly less colla- n the skins from the lean animals. r, when the collagen values were ex- as mg collagen per g lipid-free dry all differences in the collagen content from lean and obese animals disap- On a mg collagen per cm^2 of skin e skins from the obese animals again ɔd significantly less collagen than the ɔm lean animals. These data demon- at the ratio of collagen to noncollagen due (lipid-free) is the same in skins ɪn and obese animals. Therefore, the t decrease in the collagen content (mg per g wet or dry weight) of the skins ese Zucker rats probably results from ɔrmal accumulation of lipid in the her than from a defective deposition ɔollagen.

rties of collagen from skin of obese and lean Zucker rats. The data in Table III demonstrate that the ratio of acid soluble to acid insoluble collagen was the same for skins from obese and lean animals of either sex. There was a slight but significant increase in the amount of pepsin-solubilizable collagen in the skin of obese females when compared to lean females. No such increase was found for the male animals. The ratio of 1.5 *M* NaCl-soluble (Type I enriched) to 1.5 *M* NaCl-insoluble (Type III enriched) collagen in the pepsin soluble fraction was the same for all groups of animals. These data taken together with the data in Tables I and II suggest that the collagen of obese skins is essentially normal and not different from the collagen of lean skins when compared on the basis of quantity per g lipid-free dry weight, on the basis of solubility in acid, or on the basis of the ratio between 1.5 *M* NaCl-soluble and 1.5 *M* NaCl-insoluble collagen. The data demonstrating that the obese skins contain less collagen per cm^2 of full thickness skin than the lean skins suggest either that the obese skins may be thinner or that the density of collagen per cm^2 of obese skin is decreased because of displacement of collagen fibers due to the abnormal accumulation of lipid.

Break strength of skin. The break strength of skin from obese animals was significantly

TABLE II. COLLAGEN CONTENT OF SKIN FROM OBESE AND LEAN ZUCKER RATS.

nals	Age[a]	Collagen content of skin[b]			
		mg collagen/g wet wt. skin	mg collagen/g dry wt. skin	mg collagen/g lipid-free dry wt. skin	mg collagen/cm^2 skin
lean	2 months (6)	151.4 ± 4.5	463.6 ± 21.2	616.2 ± 10.5	—
	6 months (7)	262.5 ± 7.8	656.0 ± 21.0	738.9 ± 23.6	26.1 ± 1.4
obese	2 months (6)	106.8 ± 3.0[c]	182.4 ± 8.1[c]	636.8 ± 26.4	—
	6 months (7)	158.4 ± 20.1[c]	301.6 ± 63.3[c]	728.0 ± 26.3	22.5 ± 0.7[c]
an	3 months (5)	159.9 ± 8.2	476.1 ± 39.2	589.3 ± 22.6	—
	6 months (7)	248.9 ± 5.4	640.6 ± 15.5	743.2 ± 11.3	39.4 ± 2.0
ɔese	3 months (4)	117.5 ± 3.4[c]	222.7 ± 18.2[c]	615.2 ± 11.4	—
	6 months (7)	205.4 ± 12.0[c]	463.6 ± 50.8[c]	743.1 ± 13.1	29.4 ± 1.2[c]

ɪumbers in parentheses represent the number of animals in each group.
s ± SE of the mean are reported.
values for the skins from obese animals are significantly different from the values for the skins from age atched lean animals at the level of $P < 0.05$.

TABLE III. PROPERTIES OF COLLAGEN FROM THE SKIN OF OBESE AND LEAN ZUCKER RAT.[a]

Animals	mg Collagen/100 mg recovered collagen[b]			Pepsin-solubilized collagen[b] sol. in 1.5 *M* NaCl
	Acid soluble	Pepsin soluble	Insoluble	pepsin-solubilized collagen ppt. by 1.5 *M* NaCl
Female lean (6)	11.3 ± 0.7	31.4 ± 1.2	57.3 ± 1.3	5.8 ± 0.4
Female obese (4)	12.4 ± 0.8	38.0 ± 1.0[c]	49.6 ± 1.8[c]	5.2 ± 0.2
Male lean (6)	15.4 ± 1.2	35.8 ± 2.1	48.7 ± 3.2	5.8 ± 0.4
Male obese (4)	16.7 ± 1.8	33.4 ± 1.3	49.9 ± 2.7	5.8 ± 0.2

[a] These studies were done on skins from animals sacrificed at 6 months of age. The numbers in parentheses represent the number of animals in each group.

[b] Means ± SE of the means are reported. The recovery of collagen (acid sol. + pepsin sol. + insoluble/total collagen) averaged 83.3% with a range from 71.6% to 108.8%.

[c] $P < 0.05$.

TABLE IV. BREAK STRENGTH OF SKIN FROM OBESE AND LEAN ZUCKER RATS.

Animals	Break strength[a] (kg)
Female lean (3)	10.7 ± 0.7
Female obese (3)	8.0 ± 0.6[b]
Male lean (3)	17.1 ± 1.1
Male obese (3)	12.7 ± 0.7[b]

[a] Animals (males, 8 months old; females 9 months old) were sacrificed and strips of back skin 1.25 cm wide prepared. The break strength of the skins was measured as described in Materials and methods. Means ± SE of the mean are reported. Number in parentheses represent the number of animals in each group.

[b] These values for the obese animals are significantly different from the values for age and sex matched lean animals at the level of $P < 0.05$.

lower than the break strength of skin from lean animals (Table IV). These data are in agreement with those demonstrating a decreased amount of collagen per cm^2 of obese skin since the break strength of skin is, in general, a function of collagen content per unit area (25). The decreased break strength of the obese skins may explain the observation that the skin of obese animals tears and wounds more easily than the skin of lean animals.

Summary. The skins of obese Zucker rats contained per g wet weight significantly more lipid and significantly less water than did the skins from age and sex matched lean Zucker rats. The obese skins also contained less collagen than the lean skins when compared on the basis of mg collagen per g wet weight, per g dry weight, or per cm^2 of skin; however, no difference was found when the skins were compared on the basis of mg collagen per g lipid-free dry weight. The solubility properties of the collagen from obese and lean skins were the same while the break strength of the obese skins was decreased. The results indicate that skins from obese Zucker rats have a normal collagen composition but an abnormal lipid composition. The reduced break strength of the skin from obese rats, due probably to the decreased amount of collagen per cm^2 of skin caused by a displacement of collagen fibers by lipid, may explain the frequent skin tearing observed in the obese rats.

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Stimulation of Gastrin Release in Dogs by Individual Amino Acids (40072)

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Although it is well established that placing mixtures of amino acids into the stomach increases serum gastrin concentration, there are only a few studies of the effects of individual amino acids (1). In this study 21 L-amino acids were instilled into gastric fistulas in dogs and the effect on serum gastrin concentration was noted.

Methods and materials. Dogs. Two or more months before the tests were started, three dogs (18–24 kg) had a Thomas type cannula (2) placed in the stomach to form a gastric fistula.

Amino acids. Amino acids were bought from Calbiochem, La Jolla, CA. With the exception of tryptophan which was used at 50 m*M* concentration, all other amino acids were used at 100 m*M*. The osmolarity of all amino acid solutions and of the 150 m*M* NaCl control solution was adjusted to 360 mOsm kg^{-1} with mannitol and the pH was adjusted to 7.4 with 1 *M* NaOH. As a standard stimulant for acid secretion and gastrin release, 12% liver extract (Liver Concentrate Powder, Reheis Chemical, Phoenix, AR) was used at pH 7.4.

Procedure. After an 18-hr fast, the gastric fistula was opened and the stomach washed with water. The test solution was then instilled into the gastric fistula by peristaltic pump at a rate of 10 ml min^{-1} for 30 min. The solution remaining in the stomach was drained for 2 min.

Serum gastrin. Samples of blood were drawn from a leg vein at 0, 15, and 30 min after starting the infusion of the test solution. Blood was allowed to clot at 5°, serum was removed within 2 hr and stored at −20° until the assay was performed. Serum gastrin was measured by radioimmunoassay using antibody 1296 as previously described (3). The mean of the increment above basal of the 15 and 30 min values was taken as the gastrin response in each test.

Statistics. Each amino acid and liver extract was tested three times in each dog and saline was tested 12 times in each dog. The order of tests was randomized. The significance of differences between the mean response to saline and to the other test substances was tested by analysis of variance. The results in the table are expressed as means plus or minus between-dog standard errors.

Results. Only four of the amino acids caused a significantly greater increase in serum gastrin than saline: cysteine, phenylalanine, tryptophan, and hydroxyproline (Table I). The increase in serum gastrin in response to the most potent amino acids (cysteine and phenylalanine) was not significantly different from that produed by liver extract. The fluid drained from the stomach at the end of the 30-min period of perfusion with amino acids had a pH above 4.0 in all instances. The volume of fluid recovered from the stomach was significantly greater than the saline control after instillation of cysteine, tryptophan, and liver extract (Table I).

Discussion. Before radioimmunoassay of gastrin was available, it was assumed that any agent which when perfused into an antral pouch evoked secretion of acid from a Heidenhain pouch did so by releasing gastrin. Using this method, Elwin (4) found that all of the amino acids he tested, namely, glycine, α-alanine, β-alanine, sarcosine, phenylalanine, and lysine produced statistically significant stimulation of acid secretion. Similarly, Konturek and coworkers (5) found that all of the 18 amino acids which they tested by this method stimulated acid secretion but only phenylalanine produced a significant increase in serum gastrin. Using intragastric titration in human subjects, Byrne and coworkers (6) found that tryptophan and phenylalanine caused an increase in serum gastrin and stimulated acid secretion; aspartic acid, leucine, cysteine, serine, histidine, alanine, methionine, and valine stimulated acid secretion but did not cause an increase in serum gastrin; and isoleucine, lysine, arginine, glutamic acid, threonine, glycine, hydroxyproline, and

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MEAN (±SE) INCREMENT ABOVE BASAL OF
I GASTRIN AND MEAN (±SE) VOLUME
ED FROM THE STOMACH AFTER INFUSING
SUBSTANCES INTO THE STOMACH AT 10 ML
MIN^{-1} FOR 30 MIN.

ate	Increment in gastrin (pg ml^{-1})	Volume recovered (ml)
	23 ± 9	51 ± 28
	12 ± 4	62 ± 12
	20 ± 5	113 ± 32
d	19 ± 6	113 ± 41
	24 ± 9	40 ± 10
	62 ± 14*	202 ± 38*
id	22 ± 4	139 ± 32
	3 ± 7	63 ± 14
	17 ± 5	98 ± 42
	20 ± 7	52 ± 9
line	29 ± 8*	113 ± 30
	4 ± 5	72 ± 25
	12 ± 5	149 ± 26
	18 ± 5	155 ± 30
	12 ± 3	59 ± 15
ine	53 ± 19*	100 ± 17
	17 ± 8	82 ± 18
	22 ± 5	61 ± 10
	22 ± 6	79 ± 16
	39 ± 7*	293 ± 23*
	20 ± 4	66 ± 11
t	51 ± 8*	281 ± 40
	15 ± 4	86 ± 23

cantly greater than saline control, $P < 0.05$.

either stimulated acid secretion nor
n increase in serum gastrin. The
iat emerges from all of these studies
iile many amino acids stimulate acid
only a few release gastrin. The dis-
between acid secretion and release
i is probably not attributable to in-
sensitivity of the radioimmunoassay
n because earlier studies showed that
exogenous gastrin that produced
acid responses also produced de-
increases in immunoassayable gas-
lood (7). Some mechanisms other
ase of gastrin by which amino acids
mulate acid secretion include: (a)
cids may stimulate acid secretion by
a neural reflex comparable to that
y distention (8); (b) Solutions of
ids may act in part by directly stim-
he oxyntic glands they are bathing
Amino acids may release from the
and/or intestine (10) hormonal stim-
acid secretion other than gastrin.
tional hormone(s) may be related to
ut not immunoreactive to antibodies
to gastrin or may be unrelated. (d) Amino acids absorbed into the blood may stimulate acid secretion (11).

In an earlier study, we (10) found that infusion of liver extract into the duodenum of antrectomized dogs did not cause an increase in serum gastrin so we presume that the increases found in the present study are mainly of antral origin.

Although the only two amino acids which slowed gastric emptying, cysteine and tryptophan, also released gastrin, the other two amino acids which released gastrin, phenylalanine and hydroxyproline, did not affect gastric emptying. Stephens *et al.* (12) also found that tryptophan delayed gastric emptying; they could not test cystine adequately because it caused vomiting under their test conditions, an effect we did not encounter.

Summary. Solutions of 21 different L-amino acids were instilled into the gastric fistulas of three dogs. Only 4 of the amino acids produced an increase in serum gastrin significantly greater than that evoked by saline: cysteine, phenylalanine, tryptophan, and hydroxyproline.

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A Cardiomyopathy of BALB/c Mice (Superimposed Infection by Coxsackievirus B-3)[1, 2] (40073)

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During studies of murine virus myocardiopathy (1), we inoculated the Nancy strain of coxsackievirus B-3 into BALB/c mice. Unexpectedly, we observed pericarditis in normal controls. On the other hand, we had not noted these changes in virus-free Swiss mice from several sources (Albino ICR, Webster, Hauschka and Marand) or among C3H mice. We could not find an earlier description of pathologic changes in the heart in BALB/c mice (2).[2]

Here, we explore certain pathologic characteristics of BALB/c (C) cardiomyopathy. We also inoculated C mice with coxsackievirus B-3 and contrast the resultant cardiomyopathy with that described earlier in Albino Swiss ICR and C3H mice.

Materials and methods. Mice. BALB/c mice were derived by Bogg from an albino stock in 1913. The strain has been maintained continuously by brother x sister matings. C substrains from laboratories of the Michigan Cancer Foundation (C/MCF); Charles River Laboratory (C/CRL); Jackson Laboratory (C/JL); and Cumberland Laboratory (C/CL) were also used. Additionally, in several experiments noninbred Albino Swiss ICR and inbred C3H mice were employed. Rodents were fed Purina Mouse Chow and given water at will. Each strain or substrain in groups of six to ten animals was kept separately in cages. Infected mice were isolated from uninoculated controls. All mice were examined at least every third day for signs of illness.

Attempts to isolate virus from the hearts of BALB/c mice. An innate vertically transmitted virus of C mice is a possible cause for this inherent pericarditis. Primary mouse embryo fibroblast (MEF) tissue cultures were prepared from decapitated and eviscerated 14- to 18-day old Swiss Albino ICR mouse embryos. Carcasses were minced, trypsinized and grown in 30 ml plastic flasks at a concentration of 25 × 10^4/ml in Eagle's medium (EM) with 5% fetal calf serum containing 100 μg/ml of penicillin G and 50 μg of streptomycin/ml. Maintenance medium for MEF tissue cultures was EM with 2% fetal calf serum.

At several times from late term to 6 months of age, hearts of C/CRL mice were prepared for inoculation into MEF tissue cultures as 20% cell-free supernates in EM prepared with mortar and pestle; as minced tissue explants; and as single cell trypsinized suspensions (Kasten Method [3]). In each case, 0.1–0.5 ml was inoculated into two MEF 30 ml tissue culture flasks. After washing 3× in calcium and magnesium free Hanks salt solution, minces and whole cells were inoculated as 1:10 suspensions in EM. Experimental cultures and uninoculated controls were incubated (37°), and observed daily for cytopathic effects. Between the 7th and 10th days of incubation both groups of cultures showed rounding, granularity and loss of cells into supernatant fluids. Cells were then scraped from flasks, suspensions centrifuged (300 g, 4°, 5 min) and debris resuspended in EM (1:10). Again, 0.1 ml of these suspensions were inoculated into two new MEF flasks. Two similar blind passages were carried out with each specimen.

Experimental infections. Pregnant mothers were obtained from their breeding laboratories, observed and periodically sacrificed. In experimental infections, 14-day-old mice were inoculated intraperitoneally with 0.05 ml of coxsackievirus B-3 containing $10^{3.5}TCD_{50}$.

Methods for sacrificing; bleeding; autopsy; preparation of specimens for histologic examination and isolation, titration and identification of coxsackievirus B-3 *in vero* or rhe-

[1] Aided by grants from the American Heart Association (73-773) and The Skillman Foundation.

[2] This work was presented in part at the 68th Annual *Meeting of The American* Society for Microbiology, *Detroit, Michigan, May 5–10, 1968.*

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ıe cultures have been described (1, 4). us isolations with infected BALB/c e separated the right and left ventri- blunt dissection. To remove adhering ach ventricle was washed 5x by rins- ıpproximately 10 vol of sterile saline ıtted dry. The final wash was titered dual virus, and the quantity of virus nyocardium was taken as the differ- tween that in the specimen and that inal wash. At midregions of right and tricle of each heart three sections were ed for histologic changes.

ts. Pericarditis. Uninoculated mice re- well. However, during routine histo- udies of coxsackievirus B-3 myocar- veral uninoculated control C/MCF d mononuclear infiltrates in the right ılar pericardium along with minimal ardial myocardial necrosis in subja- eas. These changes were exclusively to the right heart. In some of the mice as only pericarditis, while in others, as also spotty myocardial necrosis im- ly beneath the pericardial exudate. ler to determine if these changes were to C mice bred at the Michigan Can- ndation, we obtained BALB/c mice ree other colonies, namely, substrains ; C/JL/ and C/CL. Subsequently, we d and periodically sacrificed 55 mice (from birth through 1-year of C/CRL and 14 C/JL mice (up to 90 l); 26 C/CL (just before delivery); and L mice (birth to 107 days old). Mice ree of the sources showed an identical ight ventricular pericarditis. Similar ic changes occurred in 42% F), 50% (C/CRL and 43% (C/JL) n the other hand, we found that hearts B/c mice up to 45 days old which ed at the Cumberland Laboratory rmal (Table I, A).

after, we followed C/MCF mice con- ly over two years, an interval ap- ng their normal life expectancy. hese rodents were 17, 28, 56, 110 and s old 1 of 4; 0 of 5; 5 of 5; 3 of 5; and mice had characteristic right ventric- ricarditis (Table II, A). At 10–12 of age, 3 of 10 of these mice had litis. No mouse older than a year these changes. Thus, in the C/MCF n, inherent pericarditis usually ap- peared from the second through the sixth month of life, its incidence declining there- after, and disappeared by the time these mice were a year old. We did not find acute mononuclear pericarditis in the C/CL substrain, but it is possible that pericardial changes may have been seen if we had extended our observations beyond 1½ months.

Chronic calcific fibrotic pericardial changes.

TABLE I. PERICARDITIS AMONG SUBSTRAINS OF BALB/c MICE.

BALB/c substrain	Age of mice[a] (Mos.)	No. of mice with pericarditis + No. of mice examined
A. Mononuclear pericarditis		
C/MCF	0–12	23/55 (42%)
C/CRL	1½	8/16 (50%)
C/JL	1½	6/14 (43%)
C/CL	fetal	0/26 (0%)
	0–1½	0/107 (0%)
B. Chronic fibrotic calcific pericardial changes		
C/MCF	6–24	8/42 (19%)
C/CRL	1½	0/16 (0%)
C/JL	1½	0/14 (0%)
C/CL[b]	8	6/29 (21%)

[a] C/CRL and C/JL mice were studied at 6 weeks of age; C/MCF mice were examined at several times through 24 months (see Table III). C/CL, fetuses were examined just before delivery (26 mice), at 1 week (22 mice), 2 weeks (37 mice), 4 weeks (15 mice), at 6 weeks (7 mice), and at 8 months (29 mice).

[b] Sex was noted only in the C/CL substrain at 8 months. There were 15 males. Three of each sex had right ventricular fibrotic pericardial scars.

TABLE II. AGE AT APPEARANCE OF PERICARDIAL CHANGES IN THE C/MCF SUBSTRAIN OF BALB/c MICE.

	Age of mice (Mos.)	No. of mice with pericarditis + No. of mice examined
A. Mononuclear Pericarditis	(17/30) 0.57	1/4
	(28/30) 0.93	0/5
	(56/30) 1.87	5/5
	(110/30) 3.67	3/5
	6	11/26
	10	2/5
	12	1/5
B. Chronic fibrotic calcific pericardial changes	6	2/21
	12	2/5
	13	4/10
	24	0/6

In continuing observations, we noted at autopsy gross whitish stippling over the right ventricle in a number of the older C/MCF mice. On microscopic review this older lesion was in the exact location as the acute mononuclear pericarditis, and was fibrosis with spotty calcification (von Kossa's stain). This calcifying pericardial scar was not seen in younger mice which were less than 6 months old; occurred less frequently than the acute pericardial lesions (Table II, B); and never accompanied the earlier mononuclear pericarditis.

Similar right ventricular calcific fibrotic pericardial changes were also seen in older animals from the C/CL substrain, and occurred equally in both sexes. We did not see these fibrotic scars in either the C/CRL or C/JL substrains, but our studies with these animals did not extend beyond 45 days (Table I, B).

Therefore, we found that BALB/c mice of each of four substrains showed parts of what appears to be an identical pericardial and subepicardial cardiomyopathy specifically localizing to the right heart. It begins as a fine mononuclear pericarditis, progresses to a denser infiltrate, and completes its evolution with mixed calcification and fibrosis.

Attempts to isolate indigenous viruses from the hearts of BALB/c mice. Hearts from the four brood mothers several days before term and from five C/CRL mice at each of several ages from 2–24 weeks were prepared as cell free supernates, tissue minces, or washed-trypsinized cells. In this experiment 39 mice were sacrificed. Each preparation was inoculated or cocultivated onto two MEF tissue culture flasks and cultures were observed daily for cytopathic changes. Degenerating cultures and uninoculated controls were blindly-passaged twice. No viruses were recovered.

Coxsackievirus B-3 infection in BALB/c mice. When Nancy strain of coxsackievirus B-3 was inoculated intraperitoneally into each of four substrains of C mice available to us, subclinical infection resulted. The kinetics of coxsackievirus in blood and heart was studied in C/MCF mice. There was viremia on day 1, but not on day 3. Virus was present in the heart on days 1 and 4, but was absent in most hearts by the 6th day (Fig. 1). In order to compare the capacity of the and left ventricles to support coxsackie B-3 replication, 9 additional C/CRL were inoculated and 4 days later were ficed. Mean virus titers in the right an ventricles are similar (Fig. 2).

On histologic section of hearts BALB/c weanling mice 14 days after i tion with coxsackievirus B-3, there was a calcific fibrotic pericardial scar which was limited to the right ventricle (Fig. 3 This pericardial scar in young mice rec ing from coxsackievirus B-3 myoca could not be distinguished from the inh chronic lesions of uninoculated older C

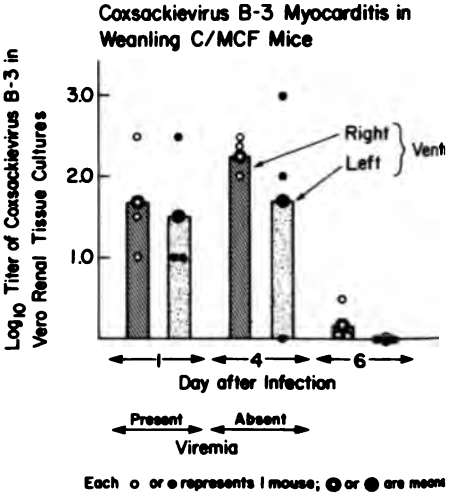

FIGURE 1

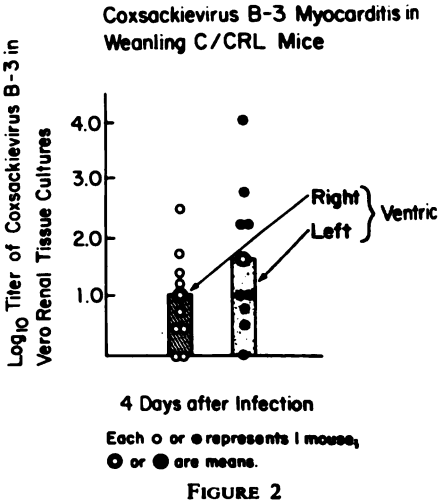

FIGURE 2

ome infected weanlings had iso-
or a more diffuse calcification in
nd right ventricular myocardium

to find the relative frequency of
and myocardial lesions in infected
ice, we inoculated 43 C/MCF, 19
C/JL and 13 C/CL 14-day-old
vith Nancy virus. All mice were
weeks later. We found right ven-
ricardial scars in each substrain,
predominant among C/MCF and
ice. Myocardial calcification was
MCF, diffuse in C/CL, and mixed
rs (Table III).

ce of Right Ventricular Pericardial
Coxsackievirus B-3 Infected
ice and in Their Controls. To de-
termine if the right ventricular calcific pericardial scars in coxsackievirus B-3 infected BALB/c mice occur more frequently in infected C mice than do similar calcified or noncalcified lesions in uninoculated controls, 29 C/MCF infected mice and the same number of age-matched uninoculated controls were sacrificed at intervals after birth from 3 to 296 days. (Calcification, of course, does not occur in the uninoculated C mice until they are at least 6 months old.) After sectioning and staining with hematoxylin and eosin, hearts were examined histologically. From 14 days onward each right ventricular pericardial lesion in infected mice contained deposits of calcium, but uninfected controls showed inflammatory exudates without mineralization through the 179th day of this experiment

FIGURE 3

id 4. Pathologic findings in weanling 4 week-old C/MCF mice 14 days after infection with the Nancy
ackievirus B-3 are shown. Sections are stained with hematoxylin and eosin (× 415). Fig. 3. A right
alcific fibrosing pericardial scar is seen. Fig. 4. Focal myocardial calcification is shown. The arrow
lcium deposit.

(Table IV). On the 296th day, two of five noninoculated mice also contained inflammatory noncalcified pericardial lesions. In both groups, pericarditis affected only the right heart.

In infected mice the cumulative prevalence of right ventricular calcific pericarditis was 35% (10 of 29 mice). Inflammatory noncalcific pericarditis at the same sites were seen in 48% (14 of 29 mice) in the age-matched controls (Table IV). Since the incidence of pericarditis is not increased in infected C mice, it is possible but not certain that pericarditis in the infected mice occurred only in those animals with a predilection for the later development of the inherent right ventricular cardiomyopathy. When coxsackievirus B-3 infection supervenes, however, it is apparent that the appearance of calcific pericarditis is markedly hastened.

Discussion. BALB/c mice have been used extensively in biological research for over 6(years, but we could find no previous repor of the spontaneous continuing right heart cardiomyopathy described here in C mice obtained from four separate breeding colonies (2). The etiology of the BALB/c lesions which we have not seen in Swiss mice from several sources, or in inbred C3H mice remains unknown. We have been unable to recover a vertically transmitted virus (5).

In about a third of several hundred BALB/c mice which we have examined and some time after the second month of life a mononuclear infiltrate appears in the pericardium of the right ventricle. This may be associated with minimal subjacent myocardial necrosis without leukocytic infiltration. Beginning after 6 months of age, these C mice have only a partially calcified pericardial scar remaining. Early and late lesions are strikingly limited to the pericardium of the right

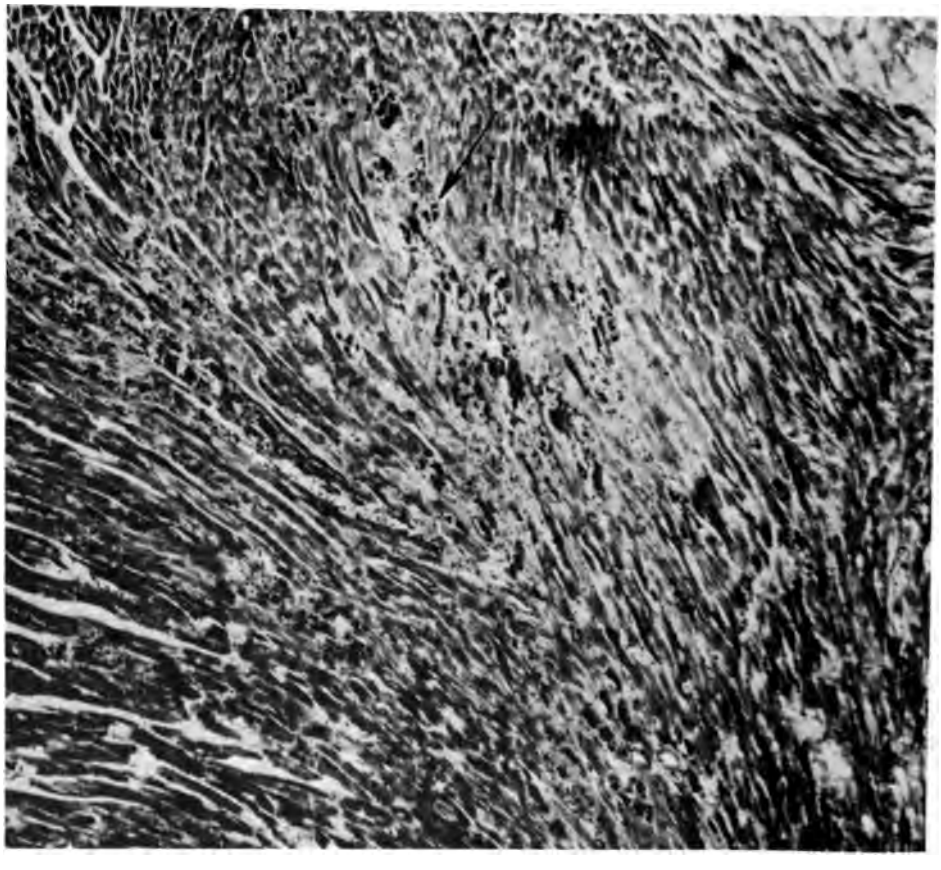

FIGURE 4

no calcification is seen before
' of life.
us pericarditis and myocarditis
tion has been reported in DBA
and, occasionally, in Swiss mice
lial calcification (without peri-
vement) has been induced in
:s of rodents including BALB/c
s, hydrocortisone or low protein
None of these changes resemble
ed here.
'irus B-3 myocarditis in Swiss or
nspicuously spares the pericar-
). Nevertheless, when BALB/c
)m four distinct breeding colo-
ılated with the Nancy strain of
s B-3, pericardial changes local-
ght ventricle, as well as myocar-
Coxsackievirus B-3 (Nancy)
equally high titers in both the
ventricles, but pericarditis oc-
he right heart. After one month
s B-3 recovered C mice have
tic pericardial scars which are
hose which can only be seen in
(6 months or more) uninfected

III. PATHOLOGIC CHANGES OF IRUS B-3 MYOCARDITIS IN SEVERAL STRAINS OF BALB/c MICE.

Histologic findings	
ricardium	Myocardium
/43* (67%)	15/43 (35%) focal
19 (47%)	5/19 (26%) focal and diffuse
5 (40%)	5/5 (100%) focal and diffuse
13 (23%)	11/13 (85%) diffuse

mice with designated finding + number
d.

siblings. The prevalence of pericarditis is not increased in infected BALB/c mice, and it may be that pericarditis develops only in BALB/c mice with a predilection for the cardiomyopathy. Coxsackievirus B-3 infection, however, remarkably accelerates the "natural" cardiomyopathy. The right heart, thus, is already "injured" when coxsackievirus B-3 infection ensues. That injured tissues harboring inflammatory exudates are more susceptible to virus infection has been noted before, but the mechanism remains unexplained (14). One may speculate that leukotactic components of complement such as C5 are present at areas of genetically determined injury in the right ventricular pericardium. Coxsackievirus B-3, complexed with antibody might be attracted to complement in the right heart, augmenting and accelerating inflammation and, ultimately, calcification (15).*

Summary. We describe progressive cardiomyopathy affecting three, and probably all four substrains of BALB/c (C) mice examined. Cardiac changes are limited to the pericardium and immediately subjacent epicardium of the right ventricle. By 2 months of age, mice have mononuclear infiltrates which progress to a dense exudative pericarditis. Whitish flecks on the pericardial surface can be seen at 6 months, and a calcifying scar persists.

When $10^{3.5}TCD_{50}$ of coxsackievirus B-3 (Nancy) is inoculated intraperitoneally into weanling Swiss or C3H mice, myocarditis without pericarditis results. If the infected mice are BALB/c, myocarditis and right ven-

* While this manuscript was being reviewed for publication, some of the original observations reported in abstract in 1968 were reported (16).

PREVALENCE OF PERICARDIAL LESIONS IN COXSACKIEVIRUS B-3 INFECTED C/MCF MICE AND THEIR AGE-MATCHED CONTROLS.

Coxsackievirus B-3 calcific pericarditis		Inherent noncalcified inflammatory pericarditis	
No. with disease + No. examined	Cumulative prevalence (%)	No. with disease + No. examined	Cumulative prevalence (%)
0 of 4 mice	0/4 (0%)	1 of 4 mice	1/4 (25%)
1 of 5 mice	1/9 (11%)	0 of 5 mice	1/9 (11%)
3 of 5 mice	4/14 (29%)	5 of 5 mice	6/14 (43%)
0 of 5 mice	4/19 (21%)	3 of 5 mice	9/19 (47%)
1 of 5 mice	5/24 (21%)	3 of 5 mice	12/24 (50%)
5 of 5 mice	10/29 (35%)	2 of 5 mice	14/29 (48%)

tricular pericarditis ensues. A calcified fibrotic scar over the right ventricle identical to the end-stage of the inherent lesion appears in coxsackievirus B3 infected mice at about one month of age.

Coxsackievirus B-3 multiplies equally well in the right and left ventricles of C mice, but pericarditis is limited to the right heart. In age-matched controls, the prevalence of calcific right ventricular pericarditis in BALB/c mice inoculated with coxsackievirus B-3 is the same as that of the inherent cardiomyopathy, and it is possible that the early calcific lesion in infected mice occurs in animals who later would have developed a right ventricular pericardial cardiomyopathy.

We thank Mrs. Mary Jane Shippey and Mr. Fred Smith for their help in these experiments.

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ulation of Immune Response in Hybrid Mice Following Rauscher Virus Infection[1] (40074)

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pression of plaque former cell (PFC) se to sheep red blood cells (SRBC) ing Rauscher leukemia virus (RLV) or leukemia virus (FLV) infection has well documented in several mouse sensitive to these viruses (1–3). Like- transient suppression of PFC response een reported for the virus-resistant /6 mouse during the first week after nfection (4). Ceglowski and Friedman mpared PFC immunosuppression by n several strains 8 days after infection :ported that the degree of immuno- ssion roughly paralleled that of virus tibility, with the only hybrid tested (the ·1 of the C57B1/6 and the DBA) show- en more immunosuppression than its ve DBA parent. However, in that study nost of the previous studies the prime n was with the immune response of known to be sensitive to the virus. r, many of these studies have been l in their observation periods to the short duration after infection in which nation of immunosuppression is usu- tained. On the other hand, recent data east one study (4) suggest that stimu- of PFC response by leukemia virus also be demonstrable in resistant if the observation period is sufficiently n the present study we have compared el of PFC response as affected by RLV sensitive SJL/J mouse, the resistant /10 mouse and their resistant hybrid- xC57B1/10)F_1. Evidence for stimu- of PFC response in the resistant mice n the second and fourth week after infection was obtained, with the immune response of the hybrid showing even greater enhancement than that of its resistant parent.

Materials and methods. Mice. The SJL/J and C57B1/10 mice were females obtained from Jackson Laboratories (Bar Harbor, ME) at the age of 6–8 weeks and quarantined before use. The F_1 hybrids were females bred in our own facilities from parents obtained from Jackson Laboratories. All mice were maintained five per cage in sterilized filter-top cages and allowed autoclaved food and water *ad libitum*.

Virus. The NB-tropic strain of Rauscher leukemia virus used was obtained from NIH in 1968 and has been maintained in our laboratories in the SJL/J mouse. Assay for the virus activity was done by a variation of the spleen enlargement dose (SED) technique of Chirigos *et al.* (6), as previously employed by us (7–9). Virus was prepared from a cell free plasma filtrate, buffered with sodium citrate and kept at −72° until use. 0.1 ml of saline diluted virus suspension containing 50 $SED_{50/14}$ units of RLV was injected ip into the mice at approximately 12 weeks of age. Control mice were sham injected with a similarly prepared material not containing virus.

PFC Response. A modification of the Kennedy–Axelrad technique (10) was used to evaluate PFC response. This technique has been shown to yield a greater number of plaques per unit of cells than the Jerne and Nordin technique (11). At the times indicated in the results, either before or after RLV administration, washed SRBC were injected into the virus infected mice and into paired age-matched control mice of the same strain. Five days later the mice were sacrificed, their spleens removed, weighed, gently mashed, and single cell suspensions prepared in PBS. Total nucleated cell number per spleen was determined and then the concentration of the

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suspension was adjusted to 2.5×10^6 cells per ml. Two milliliter of this, mixed with guinea-pig complement, was then carefully layered over a monolayer of SRBC afixed to a grided plastic Petri dish with poly-l-lysine. After a 45-min incubation at 37°, the supernate was carefully decanted off and the plates gently rinsed in PBS. The plaques per plate were counted on the damp plates and the number of PFC per spleen calculated. The plaque forming ability of the virus injected mice was compared directly with that for the normal control mice of the same strain run in parallel on each of the experimental days, and the results are reported in terms of percent of the control animal plaque forming ability.

Statistics. For each data point given a minimum of four virus infected animals was run against the controls. Most points were repeated two or more times and the data averaged in the production of the figures. Mean values and standard errors were calculated using the Student's t test as described by Dixon and Massey (12). The error limits as given in the text and figures are ± 1 SE.

Results. The mean level of PFC per spleen in the normal SJL/J, C57BL/10 and F_1-hybrid mice varied according to the strain, being 204,600 ± 9000, 293,000 ± 15,700 and 431,000 ± 24,500 respectively, which differ significantly from each other at an α level of better than 0.05. At the same time there were no significant differences in the spleen size of these normal mice, indicating that the observed variation in PFC numbers reflected a true difference in the relative proportion of plaque formers per spleen between these strains.

Figure 1 gives the data for PFC response of the virus injected animals, normalized to their respective control values for each strain and day, and presented for comparison purposes in percent of the respective controls. The RLV sensitive SJL/J mouse showed a brief elevation in response when the virus was injected 1 day before or immediately before SRBC administration. This was followed by a depression in response, reaching minimum levels at the 21st day. The C57B1/10 showed contrasting behavior, with only a slight depression in PFC response at day 2, which was much less than that previously reported for FLV infected C57B1/6 (4).

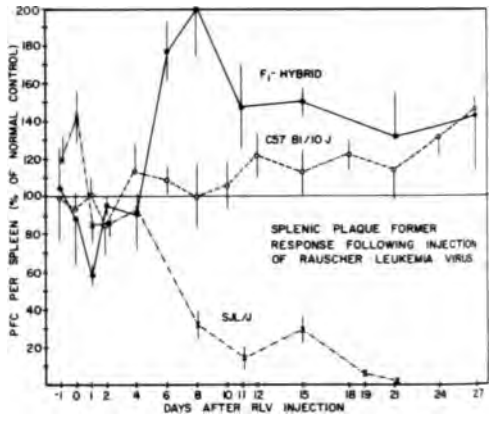
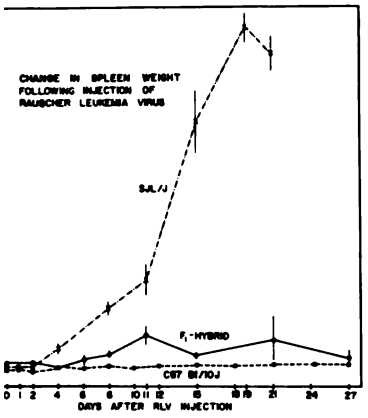


FIG. 1. Change in splenic PFC response as a function of time after RLV infection. Data is normalized to the control values for each strain taken as 100%. Error bars indicate ± 1 SE of the mean. Half error bars are shown where they would otherwise overlap.

Following this there was a recovery and overshoot which first became significant at the $\alpha = 0.05$ level at day 12. The most striking difference seen was that in the hybrid, which showed a PFC response as affected by RLV that was unlike either of its parental strains. First there was a significant immunosuppression, evident at day 1 after virus infection. This was followed by a rapid recovery in PFC, peaking at a level twice the normal by day 8, with a subsequent drop-off but a sustained immunostimulation throughout the remainder of the observation period.

Figure 2 illustrates that the observed levels of PFC response were not a direct function of spleen size following RLV infection. The C57B1/10 showed no significant change in spleen size throughout the duration of the study. The hybrid did show a transient threefold increase, but returned to normal by the termination of the study. Further, the peaks of splenomegaly for the hybrid did not coincide with the peaks of PFC activity. However, it would be correct to say that some of the increases in PFC response were due to the overall elevated number of spleen cells in the infected hybrid. By contrast, although the SJL/J developed the greatest splenomegaly, this strain showed virtually complete suppression of total splenic PFC response.

Discussion. While several authors have previously shown that murine leukemia viruses are immunosuppressive in virus-sensitive



Variation in spleen weight as a function of RLV infection. Error bars indicate ± 1 SE of Where no error bars are given the SE was less ze of the data symbols.

-3, 5, 13, 14), evidence for a possible ory effect had been limited to the 5 strain (4, 5), and only suggestive at ıe data of this paper gives positive : that enhancement of the humoral / plaque forming cell response can be ırated following leukemia virus infec- e significance of this lies in the fact noral antibody formation is known to ole in the induction of immunity to a virus in otherwise sensitive mice g the injection of killed virus and a cells (15–17). For other strains, in- the group to which the C57B1/10 it has also been established by *in* dies that one control of resistance to : oncorna virus rests directly at the the target cell, unmediated by im- sponse (18–20). In that regard the PFC response of the hybrid is of ar interest, in that the F_1 possesses or both sensitivity and resistance to s. The much greater stimulation of ormers suggests that the influence of s is more pronounced in the hybrid its resistant parent, perhaps strongly ıg an immune response to the leuke- igens. The elevated spleen weights licate an effect of the virus in the resulting in some cellular prolifera- t not to the extent observed during genesis in the SJL/J mouse, and reversible. This suggests that some ncogenic activity may have occurred which the animal was able to successfully overcome. Full leukemogenesis did not result, however, as peripheral white counts done at the terminal point of the study showed only a slight elevation, and none of the animals died of Rauscher disease. In contrast to the B6D2F_1 hybrid of the DBA and C57B1/6, which shows immunosuppression and succumbs to leukemia virus (5), we have found this hybrid survives RLV infection (21).

Because Rauscher virus is NB-tropic (22), the Fv-2 locus would be expected to play the primary role in the genetic determination of resistance to the virus. This gene governs spleen focus formation and splenomegaly, with the sensitivity allele, Fv-2^s, being dominant over Fv-2^r. The C57B1/10 is Fv-2^r (18), while the SJL/J is felt to be Fv-2^s, similar to other Swiss-Webster derived mice (18, 23). Therefore, like the B65D2F_1, one would expect this F_1 hybrid to be almost as sensitive to Rauscher virus as its SJL/J parent. Since it is not, it would seem likely that other genes in this cross may be masking the effect of the Fv-2 locus. In the related Friend virus system, Chesebro and Wehrly (24) have reported that hybrids of C57B1/10 with either BALB.B. or A.BY, which are also Fv-$2^{r/s}$, likewise show a high incidence of recovery from splenomegaly and leukemia. From their results they postulated that this may be due to other non-H-2 genes associated with the H-2^b configuration, but not found in H-2^d mice. Whether any of these other susceptibility genes are also present in the SJL/J mouse, which is H-2^s, is as yet unknown. However, if they were it might account for the observed resistance of the hybrid used in this present study to Rauscher leukemia and its recovery from splenomegaly. Additional work remains to be done to establish this point, as well as the specific response of this hybrid to the spleen focus forming virus component of the Rauscher complex (25). In so doing, this hybrid may prove to be a useful model, analogous to that of Chesebro and Wehrly (24), for the study of genetic factors governing RLV sensitivity and the development of immune response to viral leukemia antigens.

Summary. The effect of Rauscher leukemia virus injection on the splenic PFC response to SRBC was measured in a mouse strain sensitive to the virus (SJL/J), one resistant to

the virus (C57B1/10), and their F_1 hybrid (SJL/JxC57B1/10)F_1. In contrast to previous findings of a suppressive effect of Friend virus on PFC response in another hybrid of different resistant and sensitive strains, a significant immunostimulation was seen. Immunostimulation was also seen in the resistant parent when the PFC response level was measured 12 days or later after RLV infection, while the PFC response of the sensitive parent was nearly completely suppressed. Evaluation of the effect of RLV on the splenic masses of the three strains indicated a mild transient splenomegaly in the hybrid; a pronounced splenomegaly in the sensitive parent; and no effect on the resistant strain. Comparison of the data suggests that the change in splenic PFC number in the hybrid following RLV infection occurs as a function partially independent of changes in the total splenic mass.

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OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 453–455 (1978)

fect of Anionic and Nonionic Contrast Media on Renal Extraction of Para-Aminohippurate in the Dog[1] (40075)

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ɔrevious study of the renal vascular meglumine diatrizoate (1), we noted ɔn this substance was given into the ery of the dog a reversible depression renal extraction of para-aminohip- (E_{PAH}) occurred. Neither isosmotic or equiosmolar (4.5%) NaCl altered hereas both meglumine diatrizoate ⅔ NaCl caused a reversible decrease vascular resistance. The depressive meglumine diatrizoate on E_{PAH} was d to an effect of the organic iodinated ɔ on cellular transport of PAH rather ts osmotic properties.

neglumine diatrizoate was given as ıfin-76 (E. R. Squibb and Sons, Prin- IJ) which is 66% meglumine diatri- ıd 10% sodium diatrizoate. Ziegler leagues (2) reported that isosmotic ıent of serosal sodium chloride with diatrizoate depressed short-circuit and net isotopic sodium flux in the toad urinary bladder. Although basal ·cuit current was depressed, the vas- -stimulated increment in short-circuit was not affected. Separate experi- with hyperosmolar solutions (786 demonstrated equivalent suppression ·circuit current by sodium diatrizoate other solutions made hyperosmolar cose or sodium methylsulfate, imply- ɔneral or nonspecific effect of hype- rity.

the diatrizoate ion appears to depress ılium transport and PAH transport. gests that PAH transport and sodium t might be linked in some fashion. ıcentrations of sodium in the external medium have been shown to depress ke of PAH by rabbit kidney slices (3) and depress net PAH transport from bath to lumen in isolated perfused snake renal tubules (4).

Diatrizoate is an anionic single tri-iodinated ring compound. Metrizamide is a recently developed water-soluble nonionic single tri-iodinated ring compound (5). Ziegler and Olsen (6) recently reported that metrizamide did not depress basal short-circuit current in the isolated toad urinary bladder. These observations were interpreted to mean that anions (e.g. diatrizoate) may regulate sodium transport, possibly by regulating escape of transported sodium from the basolateral side of the epithelial cells.

The objective of these studies was to assess the effect of metrizamide on PAH transport in the intact dog kidney.

Materials and methods. All studies were performed on female mongrel dogs 15–25 kg in weight, fed a standard kennel ration. On the day prior to study all dogs were deprived of food but water was permitted ad libitum. On the day of the study the animal was anesthetized with intravenous sodium pentobarbitol 30 mg/kg and supplemental doses were added throughout the experiment to maintain anesthesia. The animal was intubated with an endotracheal tube and mechanically ventilated to maintain arterial pH between 7.35 and 7.45. Catheters were inserted into a femoral artery, the inferior vena cava via a femoral vein, and jugular vein to permit blood sampling, pressure measurement, and infusion of fluids. A retention catheter was placed in the urinary bladder. The left kidney was exposed via a subcostal incision and the renal artery dissected free, taking care to leave the renal nerves intact. An external electromagnetic flow probe was placed on the left renal artery and led to an electromagnetic flow meter (Carolina). This system was calibrated *in vivo* at the end of each experiment. A catheter was placed in the left renal vein via the left ovarian vein. A 25-

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gauge curved needle attached to polyethylene tubing and connected to a Harvard infusion pump was placed in the left renal artery against the direction of flow. At the conclusion of surgery a priming dose of PAH was given followed by a constant infusion of PAH in 0.9% NaCl at 1.0 ml/min to maintain a plasma concentration of 0.02 mg/ml. Following surgery a minimum of 60 min was allowed for equilibration and stabilization of solutions before beginning the experiment.

The Control Period (C) consisted of 20 min during which duplicate simultaneous samples of femoral arterial and left renal venous blood were obtained while 0.9% NaCl was being infused into the left renal artery at 3.82 ml/min. Then the left renal arterial infusion was changed to an experimental test infusion (Table I) at the same rate and following 15 min equilibration, the Experimental Period (E) was begun with sampling as noted above. Then the left renal arterial infusion was changed back to 0.9% NaCl at the same rate and following 15 min equilibration, the Recovery Period (R) was begun with sampling as noted above.

Mean arterial pressure (MAP) was measured with a pressure transducer and recorded with the electromagnetic flow meter output as renal blood flow (RBF) on a direct writing recorder (Beckman Dynograph). Renal vascular resistance (RVR) was calculated as MAP/RBF in mmHg/ ml/min. Plasma samples were analyzed for PAH by the method of Smith *et al.* (7). E_{PAH} was calculated as A_{PAH}-RV_{PAH}/A_{PAH} where A_{PAH} and RV_{PAH} are the arterial and renal venous plasma concentrations of PAH, respectively.

The data in the text, tables and figures are expressed as the mean ± SE. The Student's *t* test was used for statistical analysis of paired data within each group (8).

Results. The results are illustrated in Figs. 1–4. Mean arterial pressure was not affected by left renal arterial experimental test infusion. Figure 1 shows that, in contrast to isosmotic saline, hyperosmotic solutions, either saline or diatrizoate salts, produce a reversible renal vasodilatation (all $P < 0.01$). Control RVR was between 0.703 and 0.782 mmHg/ml/min for the four groups. Figure 2 shows that both hyperosmotic diatrizoate salts produced a reversible decrease in E_{PAH} (both $P < 0.01$) whereas neither isosmotic nor hyperosmotic NaCl affected E_{PAH}. Con E_{PAH} was between 0.713 and 0.795 for four groups.

Figure 3 shows that isosmotic solution either NaCl, anionic (Hypaque) or nonic

TABLE I. EXPERIMENTAL TEST INFUSIONS.

Solution	N[a]	Iodine, mg/ml[b]	Osmc ity mOs kg H
Renografin 76 66% meglumine diatrizoate 10% sodium diatrizoate	7	370	150
Hypaque 60 60% meglumine diatrizoate	6	282	135
Hypaque 13.4 13.4% meglumine diatrizoate	6	63	29
Metrizamide 34.8 34.8% metrizamide	6	168	29
4.5% NaCl	8	0	150
0.9% NaCl	7	0	30

[a] Number of animals studied.
[b] Manufacturer's data.
[c] Measured by freezing-point depression.

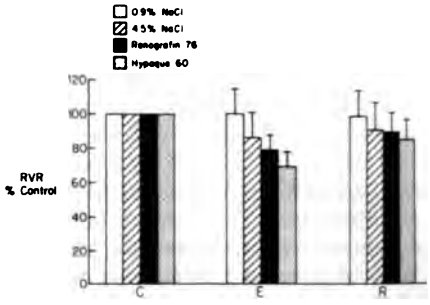

FIG. 1. Effect on renovascular resistance (RVR renal arterial infusion of hyperosmotic solutions. (control period, E = experimental period, R = reco period.

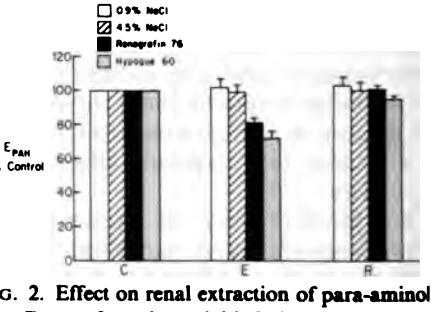

FIG. 2. Effect on renal extraction of para-aminol purate (E_{PAH}) of renal arterial infusion of hyperosm solutions.

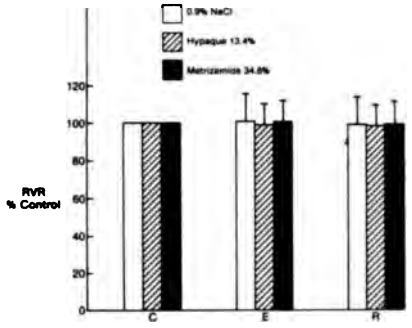

FIG. 3. Effect on RVR of renal arterial infusion of isosmotic solutions.

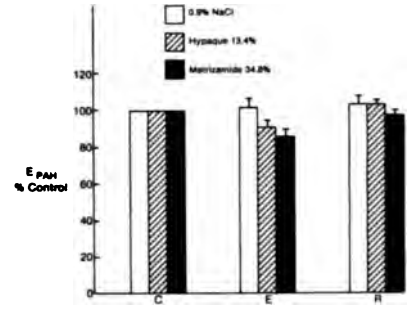

FIG. 4. Effect on E_{PAH} of renal arterial infusion of isosmotic solutions.

(Metrizamide) contrast media did not affect RVR. Control RVR was between 0.703 and 0.787 mmHg/ml/min for the three groups. Figure 4 shows that both isosmotic contrast media produced a reversible decrease in E_{PAH} (both $P < 0.05$). Control E_{PAH} was between 0.751 and 0.795 for the three groups.

Discussion. These studies demonstrate that hyperosmotic solutions infused into the renal artery produce renal vasodilation but that the depressive effect of iodinated contrast media on E_{PAH} is not related to their osmotic properties.

Since the circulating levels of PAH were about 2 mg%, the possibility that the depression of E_{PAH} was due to exceeding the maximal tubular capacity for PAH transport may be excluded. The possibility that the contrast medium competed with PAH for tubular transport and thus decreased E_{PAH} is unlikely since its excretion is almost totally by glomerular filtration (9). In our previous studies (1) Renografin did not alter intrarenal distribution of blood flow as measured with radioactive microspheres; therefore, it seems unlikely that the fall in E_{PAH} reflects a shift in renal blood flow from cortex to medulla. Since the contrast media significantly depressed E_{PAH} whether it was hyperosmotic or isosmotic and equiosmolar NaCl did not, it is clear that the effect is not due to the hyperosmotic nature of the contrast media. In accord with the studies of Danford *et al.* (10) it appears that the effect is mediated by the organic iodinated portion of the molecule.

Of interest is the fact that metrizamide depresses E_{PAH} but does not depress basal short-circuit current in the isolated toad urinary bladder. Thus, both the anionic (diatrizoate) and non-ionic (metrizamide) contrast media decrease E_{PAH} by a mechanism that is unrelated to their osmotic properties and probably dependent on the organic iodinated portion of the molecule. However, only the anionic form (diatrizoate) lowers basal short-circuit current in the isolated toad urinary bladder. These observations are in support of Ziegler and Olsen's view (6) that anions (e.g. diatrizoate) may regulate sodium transport but do not indicate that there is a dependent link between sodium transport and PAH transport.

Summary. Nonionic and anionic contrast media, isosmotic or hyperosmotic, decrease E_{PAH} while equiosmolar NaCl does not; this effect is mediated by the organic iodinated portion of the molecule.

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Enhanced Expression of Histocompatibility Antigens on Interferon-Treated Mouse Embryonic Fibroblasts[1] (40076)

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Interferon induces pronounced modifications of the surface of cells both *in vitro* (1–8) and *in vivo* (9, 10) and these changes may well be important in understanding the varied biologic effects of interferon (11). In investigating the effect of interferon on the cell surface, we showed previously that incubation of murine lymphoma and normal lymphoid cells with interferon resulted in a marked enhancement in the expression of surface histocompatibility (H-2) antigens (2, 3). Likewise inoculation of mice with interferon or interferon inducers resulted in an increase in the expression of H-2 antigens on the surface of thymocytes and splenic lymphocytes (9, 10). H-2 antigens on viral infected target cells appear to be an integral part of the recognition and destruction of these cells by cytotoxic lymphocytes (12–18). Since we have shown that interferon increases the expression of H-2 antigens on lymphoid cells and enhances the cytotoxicity of sensitized lymphocytes (19), it seemed important to determine whether interferon, a product of viral infected cells, could also modify the expression of H-2 antigens on nonlymphoid cells, or whether this effect was restricted to lymphoid cells. The results presented herein show that mouse interferon does in fact markedly enhance the expression of H-2 antigens on mouse embryonic fibroblasts.

Materials and methods. Mice. Pathogen free DBA/2, C3H and C57Bl/6 mice were obtained from the breeding colonies of the Institut du Cancer. Breeding couples of B10 and B10 D2 mice were obtained from Jackson Laboratories Bar Harbor, Maine and a colony was maintained at the Institut du Cancer.

Interferon and control preparations. Mouse interferon was prepared from C-243 cells induced with Newcastle Disease virus. The techniques of preparation, concentration and assay have been previously described (20). The control preparation consisted of medium harvested from uninduced C-243 cell cultures. The cell free supernatant was processed and concentrated in a manner identical to that used in the preparation of interferon (20). In one experiment brain interferon was prepared from mice inoculated intracerebrally with West Nile virus (21). One of our units, as expressed in the text is the equivalent of four reference units. The specific activities of the C-243-cell and brain interferon were approximately 10^6 units and 10^4 units/mg protein respectively. Human leucocyte interferon was kindly provided by Dr. K. Cantell (22) and rabbit interferon was prepared from RK_{13} cells inoculated with Newcastle Disease virus.

Sera: The origin of the sera used is summarized in Table III.

Assays of cytotoxicity. The method used for determining the specific complement dependant cytotoxicity of alloantisera on radioactive labelled chromium (^{51}Cr) target L1210 ($H\text{-}2^d$) cells have been previously described (1) as has the technique of ^{51}Cr labelling of mesenteric lymph node cells (23). The microdroplet lymphocytotoxicity tests were carried out in Terasaki plates as previously described (24).

Experimental plan. Secondary fibroblast cultures from 18-day old embryos were seeded at a concentration of 5×10^5 or 10^6 cells/ml in 3 cm diameter Falcon Petri dishes (2 ml/dish) in 5% CO_2–air at 37°. After 18–24 h in culture, the nonadherent cells were removed, the monolayer culture was washed and nutrient MEM medium with 5% calf serum was replaced containing approximately 10,000 units of mouse interferon or suitable control preparations. After 18 hr of incubation with interferon, the cell monolayer was washed 3× and incubated with twofold dilutions of 0.2 ml alloantiserum.

[1] This work was aided by the D.R.M.E. (Contract 76/342), D.G.R.S.T. (Contract 76.7.1450) I.N.S.E.R.M. (ATP 43.76-75 and CRL 77.4.072).

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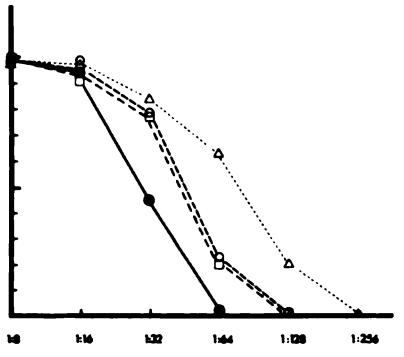
ıcubation of cells with antiserum for
e residual serum was removed and
as described above for complement
ɔnt cytotoxicity. Unless otherwise in-
determinations were always carried
uplicate.

*ts. Enhanced expression of H-2 anti-
interferon treated DBA/2 embryonic
ısts.* Secondary cultures of DBA/2
ɔmbryonic fibroblasts were treated for
vith 10^4 units of mouse C-243 cell
ɔn and then incubated with varying
s of a C57Bl/6 (H-2^b)-anti L1210 (H-
serum. As illustrated in Fig. 1, inter-



. Assay of residual C57Bl/6 anti-L1210 cell
^{51}Cr labelled L1210 cells after absorption on
secondary cultures of DBA/2 embryonic fi-
(O-----O); or cultures treated with a control
ɔn (□-----□); or with cultures treated with 10^4
'-243 cell interferon (●——●). The activity of
ıerum prior to absorption is shown by
.

feron treated cultures absorbed a greater amount of alloantiserum (see antiserum dilution 1:32) than cultures treated with a control preparation or left untreated. Another experiment using five cultures per serum dilution showed the reproducibility of the phenomenon (experiment 1 Table I).

Only treatment of cells with partially purified mouse C-243 cell interferon or crude mouse brain interferon resulted in an increased absorption of alloantiserum. Human and rabbit interferon preparations were ineffective (experiment 2, Table I) as were mouse control preparations (experiment 1, Table I).

In the above experiments sera were absorbed with 10^6 cells treated with 10^4 units of interferon but the enhancing effect of interferon on the expression of H-2 antigens was also observed when fewer cells (2.5×10^5), a smaller amount of interferon (10^3 units of interferon) were used. (Minimal dose of interferon appeared to be approximately 10^2–10^3 units).

Determination of the number of viable and dead cells in the fibroblast cultures of the different groups showed that interferon had not affected cell multiplication in the first 24 hr. This finding was in accord with previous work of Lindahl–Magnusson and coworkers who found that the inhibitory effect of interferon on the multiplication of mouse embryonic fibroblasts was not observed until after several days of culture (25).

Evidence that the effect of interferon is on the expression of H-2 antigens. To define the

; I. ENHANCED EXPRESSION OF H-2 ANTIGENS ON MOUSE INTERFERON TREATED DBA/2 EMBRYONIC FIBROBLASTS.

Antiserum[a] absorbed on 10^6 DBA/2 embryonic fibroblasts	Treatment of fibroblasts[b]	% Specific ^{51}Cr release from L1210 target cells
No	—	86% ± 11
Yes	None	78% ± 7
Yes	Control preparation	66% ± 6
Yes	Mouse C-243 cell interferon (10^4 units)	46% ± 8
No	—	100%
Yes	None	100%
Yes	Human leucocyte interferon (10^4 units)	93%
Yes	Rabbit RK13 cell interferon (10^2 units)	97%
Yes	Mouse C-243 cell interferon (10^4 units)	46%
Yes	Mouse brain interferon (10^3 units)	56%

ıl/6 anti-L1210 cell serum diluted 1:20 and 1:32 in experiments 1 and 2 respectively. Specific ^{51}Cr release
ɔd for residual antiserum after absorption on DBA/2 fibroblasts.
ɛ were five cell cultures per serum dilution in experiment 1 and duplicate cultures in experiment 2.

optimal experimental conditions for demonstrating enhancement by interferon of the expression of H-2 antigens on embryonic fibroblasts and to show that interferon itself was responsible we have used in the preceding experiments a crude C57Bl/6 anti-L1210 tumor cell serum absorbed on DBA/2 fibroblasts and assayed on ^{51}Cr labelled L1210 tumor cells. In the following series of experiments we have shown by the use of different sera, different embryonic fibroblasts and different target cells in the assays that interferon did enhance the expression of antigens coded by the H-2 complex. Firstly, the results of the experiment summarized in Panel A, Table II showed that enhancement in the expression of H-2 antigens occurs in an experimental system in which neither tumor antigens nor tumor cells were present, ie., an antiserum directed against C57Bl/6 lymphocytes. Secondly, in the experiment illustrated in Panel B (Table II) we used a commercially available monospecific anti-H-2^d serum, absorbed on DBA/2 fibroblasts and assayed on ^{51}Cr labelled mesenteric B10 D2 lymph node cells. In a second experiment (Panel C Table II) we used a crude C57Bl/6 (H-2^b) anti-DBA/2 (H-2^d) sera absorbed on B10 D2 fibroblasts and assayed on B10 D2 lymphocytes. B10 D2 mice (H-2^d) are congenic with B10 mice (H-2^b) differing from these only at the H-2 locus. Thus by testing the C57Bl/6 sera on B10 D2 fibroblasts we determined specifically expression of H-2 antigens. (Preliminary tests showed that this serum did not exhibit any toxicity for B10 lymphocytes).

Effect of interferon on the expression of H-2K, H-2D, and Ia antigens on C3H embryonic fibroblasts. We have previously observed that interferon exerted a differential effect on the expression of H-2K, H-2D and Ia antigens on splenic lymphocytes (10). Likewise as can be seen in Fig. 2, incubation of 10^6 C3H embryonic fibroblasts with 5×10^3 units of C-243 cell interferon resulted in a clear cut increase in the expression of H-2^k (Panel A), H-$2D^k$ (Panel C), H-$2K^k$ (Panel D) but exerted no effect on the expression of antigens detected by an antiserum directed against $I^kG^kS^kTL$ (Panel B).

Discussion. The results of previous experiments showed that interferon treatment of

TABLE II

	PANEL		
	A	B	C
Antiserum prepared:	*DBA/2 anti-C57Bl/6*	*B10Br anti-B10 D2 (Searle)*	*C57Bl/6 anti-DBA/2*
Dilution	1:258	1:10	1:15
Absorbed on embryonic fibroblasts from:	C57Bl/6	DBA/2	B10 D2
Antiserum assayed on target cell:	C57Bl/6 splenic lymphocytes	B10 D2 mesenteric lymph node cells (labeled with ^{51}Cr)	B10 D2 mesenteric lymph node cells (labelled with ^{51}Cr)
Results expressed as:	% of lysed cells (trypan blue)	% specific ^{51}Cr release	% specific ^{51}Cr release
Antiserum not absorbed	81% ± 12	96%	84% ± 13
Antiserum absorbed on fibroblasts —Not treated	71% ± 11	36.3% ± 5	62% ± 3
Antiserum absorbed on fibroblasts —Treated with a control preparation	68% ± 9	–	74% ± 8.5
Antiserum absorbed on fibroblasts —Treated with mouse C-243 cell interferon (10^4 u)	39% ± 5	23.3% ± 2.5	27% ± 6

TABLE III.

Laboratory of origin	Mouse strain	Directed against
Author	C57Bl/6 anti-DBA/2*	H-2^d
Author	DBA/2 anti-C57Bl/6	H-2^b
Searle Diagnostic	B10 Br anti-B10 D2	H-2^d
Dr. H. O. McDevitt	C_3H.SW anti-C3H-DiSn	H-2^k
Dr. H. O. McDevitt	(A.TL × C3H.OL) F1 anti-C3H	H-2K^k
Dr. H. O. McDevitt	(A.TL × B10.A) F1 anti-B10.Br	H-2D^k
Dr. H. O. McDevitt	A.TH anti-A.TL	I^{k}G^{k}S^{k}TL

* One antiserum was made by injecting L1210 (H-2^d) tumor cells and another by injecting normal DBA/2 splenic lymphocytes. All other sera were produced by injection of normal lymphoid cells.

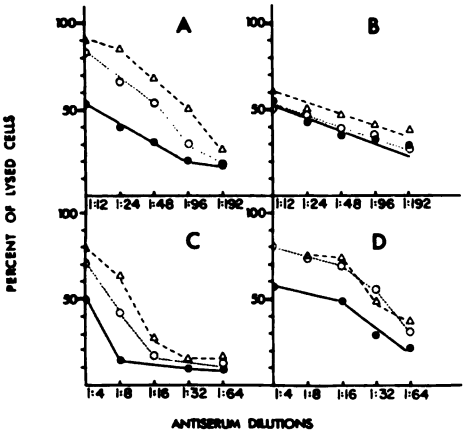


FIG. 2. Assay of residual sera on C3H splenic lymphocytes (expressed as % of trypan blue positive cells) after incubation with untreated (○——○) or interferon treated (5 × 10^3 units of C-243 cell interferon (●——●). The activity of the antiserum prior to absorption is illustrated by (△——△). Panel A, anti-H-2^k. Panel B, anti-I^{k}G^{k}S^{k}TL. Panel C, anti-H-2D^k. Panel D, anti-H-2K^k

malignant or normal lymphoid cells resulted in an increase in the expression of H-2 antigens (2, 3, 9, 10) It seemed important to us to determine whether this effect was restricted to lymphoid cells or was of more general relevance and could be observed on normal nonlymphoid cells. The results presented herein clearly show that interferon enhanced the expression of H-2 antigens on embryonic fibroblasts prepared from different strains of mice. In previous work alloantisera were often assayed on chromium labelled tumor cells and probably contained antibody to antigens other than H-2 antigens (2, 3). In this study we showed that interferon treatment was accompanied by an enhancement in the expression of H-2 antigens in experimental systems where tumor antigens were not involved either in the preparation of sera or in the assay. The use of congenic mice differing only at the H-2 locus, and monospecific and restricted sera against determinants of the H-2 complex permit us to affirm, with the confidence that these reagents and techniques allow, that we are measuring the expression of H-2 antigens, and furthermore that the effect of interferon appears to be specific for antigens coded for by the K and D region of the H-2 complex. No effect was observed on the expression of Ia antigens.

These experiments and those previously reported raise a number of questions. Does the enhanced expression of H-2 antigens on interferon treated lymphoid or non lymphoid cells reflect an increase in the amount of H-2 antigens due either to an increase in synthesis or a decrease in shedding, or a difference in the spatial presentation of H-2 antigens on the cell surface? To date we have observed an increase in the expression *only* of H-2 antigens on interferon treated cells. Interferon treatment has not resulted in any modification in the expression of Thy-1 (3), Ia (10) or TL (Lindahl, P., unpublished observations) antigens. What is the explanation for the seeming selectivity of this effect? Lastly what is the relationship between the effect of interferon on the expression of H-2 surface antigens and other effects of interferon on cells ie. antiviral action, inhibition of cell division and enhancement of cell function.

Summary. Treatment of mouse embryonic fibroblasts with mouse interferon enhances the expression of surface histocompatibility antigens as demonstrated by quantitative absorption of anti-H2 sera. Interferon exerts a differential effect on the expression of H-2D, H-2K and Ia antigens.

We are deeply indebted to Drs. H. O. McDevitt and D. Murphy for their generous gifts of antisera and their advice.

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Glycosaminoglycans of Cardiac Tumors[1] (40077)

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diac tumors are rare neoplasms that ate mostly in connective tissue (1). The : of the connective tissue, especially the aminoglycans (GAG), in heart tumors ɔt been well established, but a compar- f normal heart GAG to that of cardiac s should help reveal the histogenesis of neoplasms. In this study, several tumors ɔompared to normal human heart tissue AG composition.

'hods and Materials. Tumors were col- during surgery or from necropsy and n formalin or frozen until studied. They obtained from Charity Hospital and ı Infirmary in New Orleans, LA; Mayo in Rochester, MN; and Lafayette Med- aboratory in Lafayette, LA. Three of x tumors were myxomas, one was a nant mesenchymoma, one was a rhab- ɔma, and one was a primary angiosar- that we obtained in three parts: (a) · of the left ventricle, (b) residual tumor eft ventricular wall, (c) tumor of the atrial cavity. Control tissues were col- shortly after necropsy from portions of ee wall of the left and right ventricles he ventricular septum from Charity tal patients who did not show clinical ıce of heart disease.

'ation of GAG. The control cardiac and · tissues were minced, defatted, dehy- l with acetone, and air-dried. GAG :xtracted with 2% sodium hydroxide for from dry-defatted tissue by methods bed earlier (2). The extracts were neu- :d with 7.35 *N* phosphoric acid and ed with pronase for 72 hr while being ed against 0.1 *M* phosphate buffer pH 'he digests were then treated with tri- ɔacetic acid, filtered through celite, and ed against distilled water. The samples were concentrated, and the total content of GAG isolated from the tissue was estimated by uronic acid analysis (3).

Fractionation of GAG by column chromatography. The isolated GAG were fractionated on Dowex-1X2 (AG 200–400 mesh) Cl^- column, 1.0 × 50 cm, by eluting with a stepwise-increasing concentration of NaCl (0.5–4.0 *M*) (4). The effluent fractions were dialyzed, concentrated, and analyzed for uronic acid content. Briefly, the resin technique fractionates GAG, due to their polydispersity and varying degrees of sulfation, essentially into groups of compounds that require further chemical analyses for quantitation of individual GAG. Similar fractionations have been done on GAG preparations from a number of sources, including skin, aorta, heart, kidney, and liver (5–9). After studies of four tumors and control tissue were completed, another myxoma and the rhabdomyoma became available. Fractionations of GAG from these tissues were conducted by an automated column chromatographic procedure (10). The results of individual GAG obtained by both fractionation procedures are comparable.

Analysis of fractions. The GAG were then quantitated by additional analyses by an approach which has been described in detail earlier (11). Fractions obtained from resin columns were analyzed for uronic acids and hexosamines by gas–liquid chromatographic techniques (GLC) (12, 13). For hexosamine analysis the GAG were hydrolyzed with 4 *N* HCl for 16 hr in sealed tubes at 100–105°. We prepared trimethylsilyl (TMS) derivatives by a method previously described using hexamethyldisilazane and dimethylformamide (12). Samples to be analyzed for uronic acid were hydrolyzed with concentrated formic acid at 100–105° for 20 hr in sealed tubes under nitrogen. We prepared the TMS derivatives of uronic acid by the method of Sweeley *et al.* (14) and estimated chondroitin 4-

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sulfate (Ch 4-SO_4) and chondroitin 6-sulfate (Ch 6-SO_4) according to Mathews and Inouye (15). We further identified GAG fractions by electrophoresis on cellulose acetate in pyridine–formic acid buffer pH 3.0. Mixtures of chondroitin sulfates and dermatan sulfate obtained in the automated chromatographic procedure were analyzed for Ch 4-SO, Ch 6-SO_4 and dermatan sulfate (DS) by an enzymatic method described by Saito *et al.* (16).

Results. The amounts of total GAG extracted from control cardiac and tumor tissues are expressed in Table I as mg uronic acid per gm of dry-defatted tissue. Assuming uronic acid accounts for approximately 30% of the GAG, we multiply these values by three to estimate the average content of GAG. The control tissues, from right and left ventricles, contained similar concentrations of GAG. In contrast, the myxoma and malignant mesenchymoma tissue contained seven to ten times more GAG than the control tissues; the rhabdomyoma contained four times more; and the angiosarcoma tissue ranged from approximately one and a half to twice the amount isolated from control cardiac tissue.

An example of the multiple analyses of the different GAG fractions isolated from myxoma #1 after fractionation on Dowex Column is shown in Table II. Most of the GAG were eluted at 0.5, 1.25, and 1.5 *M* NaCl. The fraction eluted at 0.5 *M* NaCl contained glucuronic acid and glucosamine

TABLE I. TOTAL GAG CONTENT OF VARIOUS CARDIAC TUMORS.

Type of tumor	Patients' Sex and Age	Total GAG (mg uronic acid/g dry-defatted tissue)
Myxoma (#1)[a]	F 29 years	4.39
Myxoma (#2)	F 33 years	3.42
Myxoma (#3)	F 53 years	4.43
Malignant Mesenchymoma	—[c] 17 years	3.31
Rhabdomyoma	M 10 days	2.14
Angiosarcoma[b]	— —[c]	
(a) Angiosarcoma of left ventricle		0.77
(b) Residual tumor of (a) and normal left ventricle wall		0.79
(c) Angiosarcoma in right atrial cavity		1.08
Control Hearts (N = 5)	M 21–56 years	
Left ventricle		0.46 ± 0.09[d]
Right ventricle		0.51 ± 0.06[d]

[a] Myxomas were from different individuals.
[b] All angiosarcomas were from the same individual.
[c] — information not available.
[d] SE. GAG = glycosaminoglycans; N = Number of tissues

TABLE II. AN EXAMPLE OF ANALYSIS OF FRACTIONS FROM DOWEX 1 × 2 Cl^- COLUMN OF GAG FROM MYXOMA (#1).[a]

Fractions from column *M* NaCl	mg per g dry-defatted tissue	Gas-liquid chromatography: % of total hexosamine GlcN	GalN	Gas-liquid chromatography: % of total uronic acid GlcUA	IdUA	Electrophoretic identification	% of GAG fractions: HA	HS	DS	Ch 4-SO_4[b]	Ch 6-SO_4[b]
0.50	0.96	100	tr	100	tr	HA	100	—	—	—	—
0.75	0.19[c]										
1.00	0.58[c]	8.0	92.0	82.8	17.2	HS, CS/DS[d]	—	8.0	17.2[e]	25.8	49
1.25	1.32[c]										
1.50	1.20[c]										
2.00	0.13[c]	tr	100	74.0	26.0	CS/DS[d]	—	—	26[e]	13	61
4.00	0.01[c]										

[a] Thirteen milligrams uronic acid acid were applied to Dowex 1 × 2 Cl^- column and 11 mg recovered.
[b] Calculated by method of Mathews and Inouye (15).
[c] Combined for analysis.
[d] CS and DS cannot be resolved in the buffer used.
[e] Estimated from the iduronate content. Since DS also contained small amounts of glucuronate this value might be slightly lower than the actual content. Ch 4-SO_4 = chondroitin 4-sulfate; Ch 6-SO_4 = chondroitin 6-sulfate; CS = chondroitin sulfates; DS = dermatan sulfate; GalN = galactosamine; GAG = glycosaminoglycans; GlcN = glucosamine; GlcUA = glucuronic acid; HA = hyaluronic acid; HS = heparan sulfate; IdUA = iduronic acid

nd was considered as hyaluronic acid The combined fractions that eluted at .0, and 1.25 *M* NaCl contained both amine and galactosamine, and glucu- ınd iduronic acids. The fractions eluted 2.0 and 4.0 *M* contained galactosamine only hexosamine but contained both onic and iduronic acids. Based on these es as well as enzymatic analyses, indi- GAG fractions were calculated.

GAG compositions of human cardiac tissues and control myocardium are ed in Table III. HA and heparan sulfate were the major GAG found in the c tissue, although small amounts of GAG were also present. The observa- ıre similar to earlier ones of cardiac (7), and these GAG are more like those ations from medial and outer wall sec- f the aorta than like those from intimal ations of aorta. Of the myxomas stud- o had high Ch 6-SO_4 (about 40–50%) w HA (about 20%), as seen in myxomas d #2; and the other had high HA 50%), low Ch 6-SO_4 (about 20%), and S (about 5%), shown by myxoma #3. three tumors HS was present in smaller ıts than in control tissue, and about f total GAG was Ch 4-SO_4. The GAG ignant mesenchymoma tumor was sim- that of myxoma #3 with high HA and h 6-SO_4.

ontrast to these observations of myxo- nd malignant mesenchymoma, the angiosarcoma of the left ventricle and the right atrial cavity in the same heart showed the major GAG to be HA, HS, and Ch 4-SO_4 with lesser amounts of Ch 6-SO_4 and DS. While the compositions of GAG in angiosarcoma tissues from the left ventricle and right atrial cavity were very similar, the compositions of GAG from the residual tumor with normal left ventricular wall was somewhat similar to that of control heart tissues with larger amounts of HA and smaller amounts of the chondroitin sulfates. The rhabdomyoma, however, with low HA and DS and very high Ch 6-SO_4, appeared different from other tumors.

Discussion. Although the clinical and anatomic features of cardiac tumors have been described in many studies (17–19), much less is known about the biochemical changes that occur in different types of tumors or even cardiac tumors of similar histologic classification. Earlier studies have shown that HA and HS, as the major GAG found in normal myocardium, increase in hearts involved in amyloidosis (7). Certainly, variations in GAG composition occur in different cardiac structures (7, 20).

Myxoma is the most common primary intracavitary tumor; however, the exact nature of atrial myxomas is disputed. Some investigators consider them atrial thrombi in various stages of organization while others suggest they are true neoplasms (18). The myxoma is composed mostly of plump endothelial-like

TABLE III. COMPOSITION OF GLYCOSAMINOGLYCANS IN HUMAN CARDIAC TUMORS.

Type of tumor	Percent of total GAG				
	HA	HS	Ch 4-SO_4	DS	Ch 6-SO_4
a (#1)	21.8	3.8	17.1	16.1	41.2
a (#2)	19.8	—[a]	12.7	18.5	48.9
a (#3)	49.2	7.0	15.5	5.1	23.2
nt Mesenchymoma	50.2	12.1	10.0	12.7	15.0
myoma	16.7	27.5	—[a]	4.2	51.6
rcomas:					
ngiosarcoma of left ventricle	21.9	27.8	25.2	11.4	13.7
esidual tumor of (a) and normal ft ventricle wall	55.3	27.8	—	6.0	10.9[b]
ngiosarcoma in right atrial cavity	24.0	28.1	24.7	10.4	12.8
Hearts[c]					
5)	66.0 ± 15.1	24.3 ± 8.1	—	2.8 ± 1.5[d]	6.4 ± 2.1[d, b]

. detected.
udes small amount of Ch 4-SO_4.
difference in GAG distribution was observed between left and right ventricles.
standard error. Ch 4-SO_4 = chondroitin 4-sulfate; Ch 6-SO_4 = chondroitin 6-sulfate; DS = dermatan sulfate; glycosaminoglycans; HA = hyaluronic acid; HS = heparan sulfate; N = number.

or fibroblastlike cells dispersed in large amounts of an amorphous, metachromatic ground substance. An excellent review by Kelly and Bhagwat (19) of the histologic features of cardiac myxomas suggests that individual tumors may be differentiated by cellular composition (at least three types of cells were found (21)). Heath's histochemical studies (22) have suggested that a Ch 6-SO_4 is the predominant GAG in cardiac myxoma tissue. Our present studies indicate that in some myxomas the major GAG is Ch 6-SO_4, although significant proportions of other GAG are present, and in others the major GAG is HA. Studies of myxomas in other parts of the body, such as the mandible and salivary glands, suggest that these myxomas could form from cells that are morphologically similar (23–25). These observations are consistent with the occurence of myxomas as actual tumors and not organized thrombi.

Malignant mesenchymoma is a soft-tissue tumor of mesenchymal origin that is composed of tumor cells differentiating into two or more unrelated malignant forms. This tumor has areas of large vesicular cells and many tumor giant cells. These hypercellular areas blend with areas composed of interlacing bundles of spindle-shaped cells with an occasional focus of myxomatous stroma. These spindle-shaped enlongated cells are poorly differentiated and with azocarmine stain show collagen production. An occasional small focus of asteroid tissue with bony spicules is seen in the viable fibrous portions of the tumor. Most mesenchymal cells have been shown to have the ability to produce both collagen and GAG.

Rhabdomyoma is found mostly in the newborn or infant but may occur at any age. The neoplastic tissue, which is usually gathered into multiple small nodules, may comprise a solitary tumor or involve the entire heart. Microscopically, the "spider cells" are greatly enlarged, having a central nucleus and a cytoplasmic mass connected to the cell wall by cytoplasmic strands separated by large glycogen-filled vacuoles.

Angiosarcomas are true malignant neoplasms that appear to arise from angioblasts, usually from the interatrial septum. The tumor consists of ill-defined, anastomic vascular channels lined by atypical endothelial cells amassed in the lumen. Previously, neither histochemical nor chemical analyses have revealed the GAG composition of angiosarcomas, but our studies clearly demonstrate that GAG are present.

The observation that the angiosarcomas of the left ventricle and right atrial cavity in the same heart had the same relative proportions of GAG suggests both that the tumor's site of origin is not associated with any particular change in the characteristics of the GAG or their relative proportions, and that a cell similar to that of a multipotential cell, perhaps a less differentiated form of "normal" myocardial cells, make up tumors at both sites.

Unfortunately, there were not enough tumors available in this study to precisely associate their GAG makeup with cellular morphology. The present studies, however, suggest that a knowledge of GAG composition of the cardiac tumors can contribute to a better understanding of the nature of the tumors.

Summary. The glycosaminoglycan composition of six human cardiac tumors (three myxomas, a malignant mesenchymoma, a rhabdomyoma, and an angiosarcoma) and five control hearts were studied. Found in two different relative proportions, hyaluronic acid and chondroitin 6-sulfate were the major glycosaminoglycans in the myxomas, suggesting the possible existence of two biochemically different myxomas, one high in hyaluronic acid, the other high in chondroitin 6-sulfate. The glycosaminoglycans of angiosarcoma tissues were mainly hyaluronic acid, heparan sulfate and chondroitin 4-sulfate. Tissue of the left and right ventricles of the same heart had the same relative proportions of glycosaminoglycans. Biochemical studies on the connective tissue components may be helpful in understanding the nature of the tumors.

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Loss of Hepatic Monoamine Oxidase Activity Resulting from Replacement of its Coenzyme Flavin[1] (40078)

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Three mammalian mitochondrial enzymes are known to be covalently bound to flavin adenine dinucleotide (FAD). Succinic dehydrogenase (SDH) (EC 1.3.99.1) is part of the inner membrane while monoamine oxidase (MAO) (EC 1.4.3.4) is part of the outer membrane. The third enzyme, sarcosine dehydrogenase (EC 1.5.3.1) is present in the soluble or matrix space of the mitochondrion. SDH has been reported to be covalently bound to FAD from N-3 of the imidazole ring of a histidine residue to the 8α methyl group of riboflavin (Fig. 1A) (1). MAO has been shown to involve a bond between the sulfur atom of a cysteine residue in the enzyme and the 8α methyl group of riboflavin (2), while the linkage in sarcosine dehydrogenase is like that in SDH.

The two homologs of riboflavin which possess vitamin-like properties in mammals, 7-ethyl-8-methyl-10-(1′-D-ribityl)isoalloxazine (7-ethyl-8 methylflavin or 7-Et) (Fig. 1B) and 7-methyl-8-ethyl-10-(1′-D-ribityl)isoalloxazine (7-methyl-8-ethylflavin or 8-Et) (Fig. 1C) (3) have been studied for their ability to replace riboflavin in the coenzyme component of SDH. When 7-Et was the sole metabolically active flavin in the tissues of the rat, the activity of the liver enzyme was reduced as much as 90% (4) while when 8-Et was the sole flavin utilized by the tissues, the loss of activity was about 50% (5). It must be appreciated that 7-Et or 8-Et were the only flavins available to these rats; no detectable riboflavin can be found in the tissues. These flavins have, therefore, replaced riboflavin as the coenzyme group in all flavoprotein enzymes required for metabolism in these rats. When the rat was deprived of riboflavin the reduction of SDH activity was about 65% (4). However, rats receiving either of the homologs are indistinguishable with respect to rate of growth, efficiency of food utilization, physical appearance and survival from rats receiving riboflavin (3, 6) while riboflavin-deficient rats show extreme abnormalities with respect to these criteria and invariably die. The indispensability of SDH for life, and the absence of riboflavin in the tissues of the homolog-fed rats, suggests that the two homologs must be covalently linked to the enzyme, forming holoenzyme analogs. The bonds most likely to be formed are illustrated in Fig. 2. We have discussed these structures in earlier publications (5, 7).

7-Et possesses an 8 α methyl group as in riboflavin yet it is not as well utilized by SDH as is 8-Et in which the link is assumed to be at the α carbon of the ethyl group (Fig. 2). A similar covalent bond is likely to be required to produce a functional MAO. If the S of a cysteine residue replaces the N-3 of a histidine residue (Fig. 2) one can visualize the form of bonds required, if the homologs are to function as coenzymes for MAO. These considerations prompted us to study the rate of change of MAO activity during the time when liver mitochondrial riboflavin is being lost due to riboflavin deprivation (Study I), and the rate of change of MAO and SDH during the replacement of riboflavin by one or the other of the homologs (Study II). The results of those investigations suggested a comparison of L-DOPA metabolism by rats utilizing riboflavin with rats utilizing one or the other of the homologs (Study III).

Materials and methods. Weanling female rats of the Wistar strain,[4] initially weighing between 35 and 45 g were used for the several

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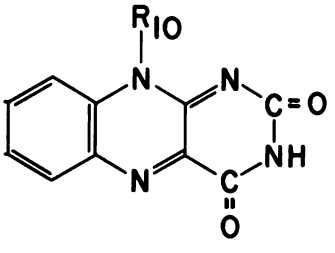
'he basic diet of the rats (feeding time "0" in all studies) consisted of .avin-deficient diet previously de-); this diet was used for the deficient roup Def) in Studies I and III. To diet was added 20 mg/kg of ribo-Group Rb only (all Studies), 20.8 7-Et for Group 7-Et only (Studies) and 20.8 mg/kg of 8-Et for Group dies II and III). The animals had s to food and water and they were l maintained as described before (9). Groups D and Rb each consisted of ng rats. At time "0" and days 1 and ere taken from each group and the ıch rat assayed independently. The cedure was carried out on days 13, , 28, 29; 41, 42, 43 and 55, 56, 57. dependent values were obtained for ıp for each time interval (Table I). Group Rb consisted of 36 and each s 7-Et and 8-Et of 33 rats. The of the MAO assay using kynura-ubstrate used in Study I and our experience with the assay for SDH permitted the use of fewer assays than were made for Study I. At the start of day 1, equal portions of the liver of three rats from each Group were pooled by Group and duplicate assays performed on each such pooled sample. The same procedure was performed on days 14, 28, 42 and 56. On days 28 and 56, three additional rats from each Group were taken for MAO assays using 1-[^{14}C]-tyramine as substrate. On days 57, 58 and 59, three rats from each Group were taken for MAO assays using 1-[^{14}C]-dopamine, 2-[^{14}C]-serotonin and 2-[^{14}C]-tryptamine, respectively, as substrates (Table I). Study III: Groups Rb, D, 7-Et and 8-Et consisted of 44, 41, 34 and 36 rats, respectively. At time "0", 10 rats from Group Rb were given interperitoneal injections of L-DOPA (320 mg/kg of body weight) (10). The same procedure was used for 10–16 rats from each Group on days 14, 28 and 56. Rats able to metabolize L-DOPA and its products survived while those unable to do so, died within 4 hr from the time of injection (Table III).

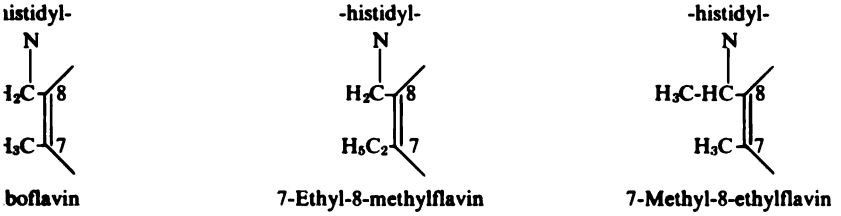
Prior to the assays for MAO and SDH, the rats were fasted for 16 hrs, then lightly anesthetized and killed by decapitation. The livers were removed and placed in cold 0.25 *M* sucrose. For Study I, tissue (2 g) was taken from the right median lobe from each rat of Group Rb, but because of their small size for all rats at time "0" and days 1 and 2, and all those from Group D at later times as well, portions from more than one lobe were required to obtain 2 g. For Study II, approximately 0.7 g portions taken from each of the three rats of a Group were pooled (2 g total).

Isolation of mitochondria. In all cases a 10% homogenate in 0.25 *M* sucrose was prepared by using 10 slow passes of the Teflon pestle



Basic Flavin Structure:

R_7	R_8	Trivial Name
H_3—	CH_3—	Riboflavin
H_5—	CH_3—	7-Ethyl-8-methylflavin or 7-Et
H_3—	C_2H_5—	7-Methyl-8-ethylflavin or 8-Et



'orms of the covalent bond between riboflavin and the homologs and SDH. The structure of the bond ıethyl-8-ethylflavin and histidine as shown is hypothesized on the basis of the rule of hyperconjugation fies the most active hydrogen atoms as those *alpha* to a double bond or other electron-deficient unit (19).

TABLE I. MONOAMINE OXIDASE ACTIVITY OF HEPATIC MITOCHONDRIAL PREPARATIONS FROM RATS RECEIVING RIBOFLAVIN (Rb), NO RIBOFLAVIN (Def), 7-ETHYL-8-METHYLFLAVIN (7-Et) OR 7-METHYL-8-ETHYLFLAVIN (8-Et). (STUDIES I AND II).

Group / Substrate	Days 0	14	28	42	56
Riboflavin (Rb)					
kynuramine	100[a]	100	100	100	100
1-[^{14}C]-tyramine			100[c]		100
1-[^{14}C]-dopamine					100
2-[^{14}C]-serotonin					100
2-[^{14}C]-tryptamine					100
Riboflavin deficient (Def)					
kynuramine	100[b]	49	29	16	16
7-Ethyl-8-methylflavin (7-Et)					
kynuramine	100	34	20	19	18
1-[^{14}C]-tyramine			39[c]		22
1-[^{14}C]-dopamine					29
2-[^{14}C]-serotonin					35
2-[^{14}C]-tryptamine					30
7-Methyl-8-ethylflavin (8-Et)					
kynuramine	100	15	6	4	3
1-[^{14}C]-tyramine			16[c]		11
1-[^{14}C]-dopamine					21
2-[^{14}C]-serotonin					23
2-[^{14}C]-tryptamine					7

[a] All assay values determined as n moles substrate oxidized/min/mg protein. The normal values for the enzyme activities in Group Rb were: time "0", 4.2 ± 0.12; day 14, 4.4 ± 0.14; day 28, 4.0 ± 0.11; day 42, 4.2 ± 0.20; day 56, 4.2 ± 0.20. All values in the table are expressed as percentage of the value for Group Rb done in parallel with the experimental Groups.

[b] The assay values for the enzyme activity for Group Def were: time "0", 4.2 ± 0.12; day 14, 1.7 ± 0.12; day 28, 1.1 ± 0.02; day 42. 0.67 ± 0.02; day 56, 0.66 ± 0.04. The "P" value for the difference between Group Rb and Group Def for each time interval is less than 0.0001.

[c] The assay values for the enzyme activity for this time interval were: day 28, Group Rb, 8.80 ± 0.36 m U/mg; for Group 7-Et, 3.41 ± 0.18 m U/mg, and for Group 8-Et, 1.38 ± 0.06 m U/mg, where one m U = 1 nmole/min. The other assays by the radiometric procedure were of comparable precision.

TABLE II. SUCCINIC ACID DEHYDROGENASE ACTIVITY OF HEPATIC MITOCHONDRIAL PREPARATIONS FROM RATS RECEIVING RIBOFLAVIN (Rb), 7-ETHYL-8-METHYLFLAVIN (7-Et) OR 7-METHYL-8-ETHYLFLAVIN (8-Et). (Study II).

	Days 0	14	28	42	56
Riboflavin	100[a]	100	100	100	100
7-Et	100	67	34	24	19
8-Et	100	71	64	58	49

[a] Assay values determined as μmoles succinate oxidized/min/mg protein. The normal value for hepatic mitochondrial preparations found in this study was 0.397 ± 0.026 μmoles/min/mg protein. All values are expressed as percentage of the value for the Rb Group done in parallel with the experimental Groups.

TABLE III. SURVIVAL OF RATS RECEIVING RIBOFLAVIN (Rb), NO RIBOFLAVIN (Def), 7-ETHYL-8-METHYLFLAVIN (7-Et) OR 7-METHYL-8-ETHYLFLAVIN (8-Et) FOLLOWING THE AMINISTRATION OF L-DOPA.[a] (Study III).

	Days 0	14	28	56
Rb	0/10[b]	0/10	1/10[c]	0/14
Def		0/10	0/11	1/20[d]
7-Et		0/10	3/10	1/14
8-Et		3/10	10/10	16/16

[a] L-3,4-Dihydroxyphenylalanine administered IP, 320 mg/kg body weight. All deaths occurred in 4 hr or less except as indicated (see d below). No hyperactivity observed.

[b] Number of rats that died over number used.

[c] This rat was found to have bled into the abdomen, probably as a result of an injury during injection.

[d] Died between 20 and 24 h following injection.

in a Thomas "tissue grinder" (size C) at 450 rpm, with the homogenizer immersed in ice and water. The tissue preparation was cooled at all steps of the procedure. The homogenate was filtered through four layers of No. 80 gauge cheese cloth. The filtrate was centrifuged at 600*g* for 10 min, the supernatant suspension was saved and the pellet resus-

ed in 18 ml of 0.25 *M* sucrose in the
ial homogenizer by gentle hand rotation
pestle. This suspension was centrifuged
ove and the combined supernatant sus-
ons centrifuged at 9000*g* for 10 min. The
natant solution was discarded and the
transferred to a 15 ml Dounce homog-
r[5] with 20 ml of 0.25 *M* sucrose. Six slow
s were made with the tight-fitting pestle
e B) and the suspension centrifuged at
for 10 min. The pellet obtained from
st centrifugation was resuspended in 10
f 0.25 *M* sucrose in a 7 ml Dounce
genizer as described before. This mito-
drial preparation was used undiluted for
assay and was diluted 1–20 with 0.25
crose for SDH assay. MAO activities
determined using either kynuramine as
rate for a spectrophotometric assay (11)
C]-labeled amines for a radiometric pro-
e (12). The SDH activities were assayed
ie use of phenazine methosulfate and
orophenolindophenol as the electron ac-
r system (13). Protein concentrations of
itochondrial preparations were deter-
d by the procedure of Lowry *et al.* (14).

sults and discussion. The rate of change
H activity when the rats consumed diets
emented with one or the other homolog
e II) was essentially identical with our
r findings (4, 5). We here report the
of hepatic mitochondrial preparations
a spectrophotometric procedure; previ-
we assayed liver homogenates by a
metric procedure. The importance of
onfirmatory information can be appre-
l when it is realized that the homolog 7-
ich yields the least enzyme function for
, is the one in which the covalent bond
8 α carbon could be established as with
-containing SDH. The homolog 8-Et
provides a far greater coenzyme func-
or SDH, is the one in which the covalent
at the 8α carbon position would need
formed by using the α carbon of an
group, a structure which must be con-
d abnormal in relation to the normal
bond but mandatory in a physicochem-
ense (Fig. 2). Further it was important
nfirm the validity of our earlier obser-
as because of the obvious normal ap-
nce of the rats receiving 7-Et, even when
they had received the homolog for hundreds of days at which time the SDH activity did not exceed 9% of the normal value.

The rate of change in MAO activity when rats consumed the flavin-free diet or the flavin-free diet supplemented with one or the other of the homologs (Table I), showed the activity was severely depressed when no flavin was available or when 7-Et was the sole metabolically active flavin, and to be vertically abolished (depending on the substrate used) when 8-Et was utilized. The reduction of hepatic MAO activity during the development of riboflavin deficiency had been studied by others (15) but the rate of fall was not as rapid as we have observed. The MAO activities when kynuramine or tryptamine (and possibly tyramine) was used as substrate, appear to reflect the degree of normalcy of the structure of the covalent bond at the pseudo-FAD junction. However, it is difficult to draw a distinction between the homologs when dopamine or serotonin is used as substrate, suggesting that the observed changes in hepatic MAO may not be true of brain MAO. These findings might also be interpreted as providing some supportive evidence for the existence of more than one form of hepatic MAO. However, the multiplicity of species of MAO is not accepted by all workers in the field (16) and for this reason it would be inappropriate to attach too much significance to variations shown among the substrates. Most investigators of MAO believe that the use of substrates to distinguish between species of MAO is not dependable while some differences appear to be demonstrable by the use of specific inhibitors such as clorgyline and deprenyl.

Our findings show that in contrast to observations with SDH, rats utilizing 8-Et for their flavoprotein coenzymes lost hepatic MAO activity. This led us to consider means by which we could demonstrate this loss of activity in the whole animal. A direct test appeared to be an evaluation of the ability of the rats to metabolize the metabolic products (presumably principally dopamine) from a dose of L-DOPA that can be tolerated by normal rats (10). When the sole flavin available to the rat has been Rb, 7-Et or 8-Et for a period of only 28 days, the animal's weight will have increased to four times the initial weight and the mitochondrial turn-over will

ntes Glass Co., Vineland, N.J.

have completed at least two half lives (4). By this time, the rats receiving 7-Et and 8-Et will have had all ionically bound Rb forms displaced form their tissues and the mitochondrial covalently bound FAD cannot exceed 3% of its original concentration. When the homologs have been fed for 56 days, riboflavin is no longer detectable in the tissues. In contrast to this, rats maintained on a riboflavin-deficient diet usually do not double their weight with the end result that after 28 days the residual total body Rb is present at approximately 55% of its original concentration (17). When the flavin available to the rats for 28 days has been 8-Et, they could not survive the administration of L-DOPA, while the rats receiving riboflavin did, and further, those receiving no flavin or 7-Et also survived in spite of the severe depression of MAO activity in the latter two cases. This raises an interesting paradox. When dopamine was used as substrate, the MAO activities of rats fed either of the two homologs appeared to be approximately the same (Table I). Yet the product of the metabolism of L-DOPA (presumably dopamine) could not be tolerated by the rats utilizing 8-Et but could be tolerated by those utilizing 7-Et. These observations warrant an investigation of the influence of these homologs on brain MAO activity. However, other amine oxidizing systems may be involved in these differences. These findings also suggest that hepatic MAO activity is not essential for the survival of the unstressed laboratory rat.

Our observations that 7-Et supports moderate MAO activity while 8-Et supports virtually none when kynuramine and possibly tryptamine and tyramine are used as substrates is in sharp contrast to our findings relative to SDH. In the latter case, 7-Et supports only very low SDH activity while 8-Et supports appreciable activity. There can be no doubt that the homologs are bound to SDH since the rats utilizing them are, as cited before, indistinguishable from those using riboflavin. These findings might be interpreted as suggestive of the possibility for the 8α methyl binding to the N-3 of histidine and the -S- of cysteine while the α-carbon of the ethyl group can bind to the N-3 on histidine only. Yeasts also possess SDH. Based on the use of samples of the homologs provided by us, it has been reported that the homologs are bound to the SDH of the mitochondria of a riboflavinless mutant of *Saccharomyces cerevisiae* (18). However, the apparent nonessentiality of hepatic MAO activity for the laboratory rat leaves some uncertainty concerning the binding of 8-Et to the hepatic enzyme. Clearly 7-Et is covalently linked to MAO however, the loss of MAO activity when 8-Et is utilized might be due to failure of the homolog to bind or, if the bond is established failure of the holoenzyme to be catalytically active.

The binding of these two homologs to SDH and MAO and the influence of these homologs on brain mitochondria are under investigation in our laboratory at present.

Summary. When 7-methyl-8-ethyl-10-(1′-D-ribityl)isoalloxazine (7-methyl-8-ethylflavin or 8-Et) is utilized in place of riboflavin in rat tissues, its coenzyme function for hepatic MAO is so low that enzyme activity is virtually eliminated. The present evidence suggests that 8-Et cannot be covalently bound to the enzyme. The absence of hepatic MAO activity when 8-Et is utilized is confirmed by the inability of rats using this flavin to survive the stress of administered L-DOPA. The use of 7-ethyl-8-methyl-10-(1′-D-ribityl)isoalloxazine (7-ethyl-8-methylflavin or 7-Et) causes a severe decrease in hepatic MAO, however, the activity of the enzyme is great enough to provide evidence that 7-Et is covalently bound to the enzyme. Rats utilizing 7-Et as well as riboflavin-deficient rats are able to survive the stress of administered L-DOPA. Hepatic MAO may not be an essential enzyme for survival of the laboratory rat.

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Effect of Antipyretics on group A Streptococcal Pyrogenic Exotoxin Fever Production and Ability to Enhance Lethal Endotoxin Shock (40079)

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Like endotoxin, group A streptococcal pyrogenic exotoxins (SPE) produce fever when given iv into rabbits (1–4). Other biological properties associated with the three antigenically distinct SPE types, designated A, B, and C, include: enhancement of the susceptibility of rabbits, mice, and monkeys to lethal endotoxin shock (2, 5, 6); alteration of reticuloendothelial function (7, 8) and the antibody response to sheep erythrocytes in rabbits and mice (9–12). These important biological properties have been reviewed (13).

Administration of pyrogens, such as endotoxin, lipid A, or endogenous pyrogen, either into the hypothalamus or intravenously produces fever which can be inhibited by the antipyretics indomethacin and acetylsalicylate (14–21). These antipyretic agents have been shown to inhibit the synthesis, and therefore, subsequent release of prostaglandins (22). Prostaglandins of the E series, particularly PGE_1 and PGE_2, have been shown to elevate body temperature in rabbits and have been implicated as hypothalamic mediators of fever (14, 20, 21). Other studies have shown that indomethacin, if given before endotoxin, can either abolish or delay characteristics associated with endotoxin shock (23, 24).

The antipyretic effect of cortisone also may involve prostaglandins since cortisone has been shown to interfere with prostaglandin release (25). In addition, cortisone may prevent fever by inhibiting the release of endogenous pyrogen from leukocytes (26–30).

This study was undertaken to determine the effects of the antipyretics indomethacin, acetylsalicylate, and cortisone on the fever response of rabbits to SPE type C and on the ability of SPE type C to enhance susceptibility to lethal endotoxin shock.

Materials and methods. Purified preparations of SPE type C, containing 25% hyaluronic acid as a stabilizing agent, were made as described previously (1, 2, 4). The minimum dose of toxin given iv to produce an average fever response of 0.5° at 4 hr post-injection (MPD-4) was approximately 1.0 μg/kg rabbit body weight. Doses of toxin used were 20 MPD-4/kg (20 μg/kg). Toxin concentrations were determined using the microbiuret protein assay described by Zamenhof (31).

Biological assays. Solutions of SPE were prepared in sterile pyrogen-free phosphate-buffered saline (0.005 *M* phosphate buffer pH 7.0, plus 0.15 *M* NaCl) for all biological assays. American Dutch rabbits 1.0–1.5 kg were used to determine pyrogenicity and enhanced susceptibility to lethal endotoxin shock (2). Pyrogenicity was measured as described previously (2). The average starting temperatures of the rabbits ranged from 37.6 to 38.1° with mean differences of 0 to 0.3° between groups in an experiment. Enhanced susceptibility to endotoxin shock was measured by giving an iv injection of 25 μg/kg of endotoxin (*Salmonella typhimurium*) 4 hr after SPE injection. The LD_{50} of endotoxin for normal rabbits was 535 μg/kg (1).

Drugs. Indomethacin was generously provided by Merck, Sharp and Dohme (Merck and Co., Inc., West Point, PA) and suspended in phosphate-buffered saline (PBS) at a concentration of 25 mg/ml. Acetylsalicylic acid was purchased from Sigma Chemical Co., St. Louis, MO. The acetylsalicylic acid (8 g in 20 ml PBS) was neutralized by adding 8 *M* sodium hydroxide and the volume adjusted to 40 ml with PBS. Cortisone was obtained from The Upjohn Co., Kalamazoo, MI.

Statistical analyses. The difference in mean fever responses between groups was evaluated at the 4 hr time point or 1 hr after drug injection, using Student's *t* test analysis. The variability in fever responses is indicated by ± SEM. Statistical analyses were performed only at the 4-hr time point or 1 hr after injection of antipyretic agent.

Results. Figure 1 shows the effect on pyr-

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enicity of treating rabbits with indomethin (25 mg/kg body weight) immediately fore administration of SPE type C. hereas control rabbits showed an average ver response of 1.1° at 4 hr, no average ver response was obtained for the indoethacin-treated rabbits ($P < 0.001$). Preatment of rabbits with indomethacin, hower, did not prevent enhancement of lethal dotoxin shock by SPE type C (Fig. 1). For e groups, either four of five or five of five bbits died less than 8 hr after treatment th endotoxin.

When indomethacin (25 mg/kg body ight) was given near the time of highest ver response, again the fever was reduced ig. 2). A drop in average fever response of ° in less than 1 hr was obtained ($P <$ 05).

Administration of acetylsalicylate (200 g/kg) before giving SPE type C (Fig. 3) o effectively prevented the fever response < 0.005). However, as observed with inmethacin treatment of animals, acetylsalilate did not protect against enhanced sus-

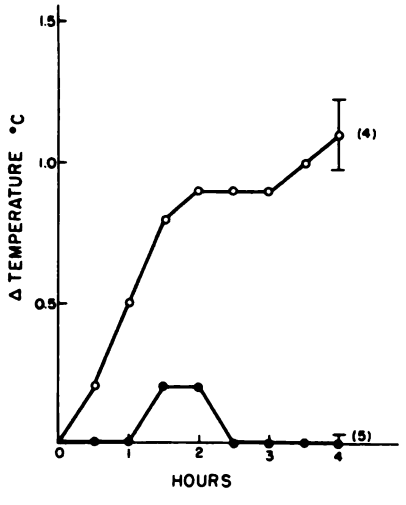

FIG. 1. The effect of indomethacin pretreatment of bits (25 mg/kg) on SPE type C fever production and lity to enhance lethal endotoxin shock. Phosphatefered saline followed by SPE type C (○); indomethn followed by SPE type C (●). Doses of SPE type C re 20 μg/kg. *Salmonella typhimurium* endotoxin (25 /kg) was given to all rabbits at 4-hr time point. Bars licate ± 1 SE. Numbers in brackets are number of the e rabbits in each group that died when given endo-in.

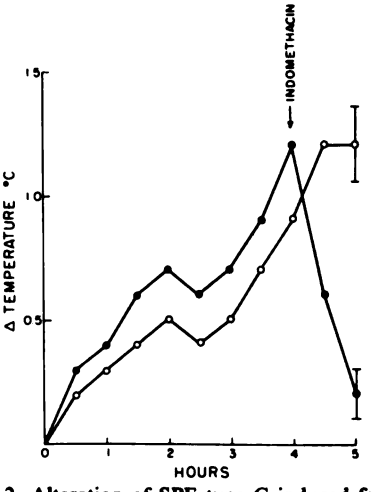

FIG. 2. Alteration of SPE type C induced fever response in rabbits by indomethacin (25 mg/kg). SPE type C followed by phosphate-buffered saline given at 4 hr (○); SPE type C followed by indomethacin given at 4 hr (●). Doses of SPE type C were 20 μg/kg. Bars indicate ± 1 SE.

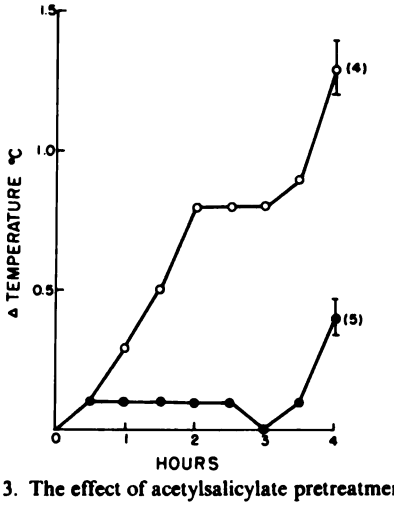

FIG. 3. The effect of acetylsalicylate pretreatment of rabbits (200 mg/kg) on SPE type C fever production and ability to enhance lethal endotoxin shock. Phosphate-buffered saline followed by SPE type C (○); acetylsalicylate followed by SPE type C (●). Doses of SPE type C were 20 μg/kg. *Salmonella typhimurium* endotoxin (25 μg/kg) was given to all rabbits at 4 hr time point. Bars indicate ± 1 SE. Numbers in brackets are number of the five rabbits in each group that died when given endotoxin.

ceptibility to lethal endotoxin shock. Four of five rabbits died in the control group, and five of five rabbits died in the acetylsalicylate-treated group. The fever response induced by

SPE type C was reduced by giving acetylsalicylate (200 mg/kg) near the time of maximum fever response (Fig. 4). A drop in temperature of 0.9° in 1 hr was obtained ($P < 0.005$).

Treatment of rabbits on each of 3 days prior to toxin with 5 mg/kg cortisone and again 2 hr before giving toxin depressed fever production by SPE type C (Fig. 5) ($P < 0.005$). Again, cortisone did not protect the rabbits from enhanced susceptibility to lethal endotoxin shock (Fig. 5). In both groups four of five rabbits died.

Discussion. Several studies have implicated prostaglandin involvement in fever production by endotoxin, Lipid A, or endogenous pyrogen (14–21). The prostaglandin involvement is based on the observations that the antipyretics indomethacin and acetylsalicylate, which inhibit prostaglandin synthetase, inhibit fever production. In the present study, these antipyretics also inhibited fever production by SPE type C, suggesting SPE-induced fever may require prostaglandin synthesis. Continuous synthesis and release of prostaglandin may be necessary to maintain SPE-induced fever since administration of antipyretics during fever also depressed the fever response.

Previously, it has been suggested that cortisone may inhibit fever production by interfering with the release of endogenous pyrogen

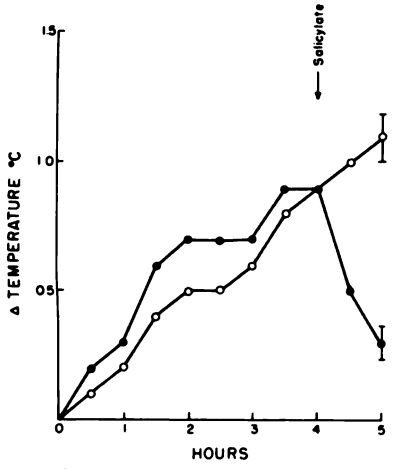

FIG. 4. Alteration of SPE type C induced fever response in rabbits by acetylsalicylate (200 mg/kg). SPE type C (20 μg/kg) followed by phosphate-buffered saline given at 4 hr (○); SPE type C followed by acetylsalicylate given at 4 hr (●). Bars indicate ± 1 SE.

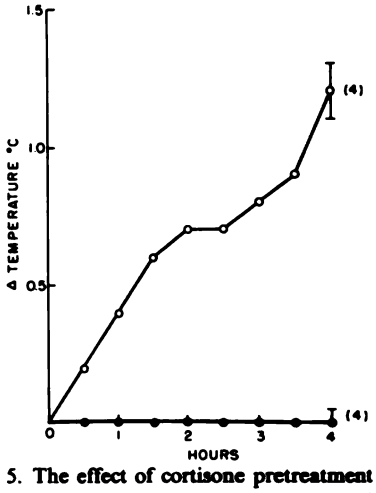

FIG. 5. The effect of cortisone pretreatment of rabbits on SPE type C fever production and ability to enhance lethal endotoxin shock. Rabbits given 20 μg/kg of SPE type C (○); schedule of cortisone pretreatment was: day (−3) 5 mg/rabbit; day (−2) 5 mg/rabbit; day (−1) 5 mg/rabbit; day (0) 5 mg 2 hr before SPE type C. Rabbits pretreated with cortisone and then given SPE type C (●). *Salmonella typhimurium* endotoxin (25 μg/kg) was given to all rabbits at 4 hr time point; numbers in brackets are the numbers of the rabbits in each group that died when given endotoxin. Bars indicate ±1 SE.

from leukocytes (26–28). The fever response of rabbits to endogenous pyrogen is characterized by a 1–2 hr maximum (29, 30), while SPE type C typically produces a gradual rise in fever which peaks near 4 hr. Therefore, SPE may produce fever through a mechanism other than endogenous pyrogen. It is possible that cortisone inhibited fever production by SPE type C through blocking the release of prostaglandin as suggested by a previous study (25) rather than by blocking endogenous pyrogen release. It cannot be discounted, however, that SPE type C acts slowly on leukocytes to induce the release of endogenous pyrogen, resulting in the 4 hr maximum.

A biologically important property of the SPE is their ability to enhance the susceptibility of animals to lethal endotoxin shock (5, 6). The mechanism underlying this property remains unclear. It may, however, be separable from fever production since abolishing fever with antipyretics did not prevent the enhancement phenomenon; control and experimental rabbits died at similar rates and numbers. Further experiments using SPE

C and endotoxin doses closer to the LD_{50}
l to be done to assess fully the role of
taglandins in the enhancement phenom-
ı and to assess the ability of antipyretics
ıodify the enhanced susceptibility to le-
endotoxin shock as previously observed
endotoxin in the absence of SPE (23,

ummary. The fever response of rabbits to
ıp A streptococcal pyrogenic exotoxin
3) type C was effectively reduced by pre-
tment of the animals with the antipyretics,
ımethacin (25 mg/kg), acetylsalicylate
mg/kg), and cortisone (5 mg on each of
e preceding days and 2 hr before given
). Indomethacin and acetylsalicylate also
ificantly reduced the fever response of
ıits to SPE type C if given near the time
ıaximum fever response (4 hr after SPE).
e of the antipyretic agents protected the
ıits from SPE's capacity to enhance sus-
ibility to lethal endotoxin shock.
ıese data suggest that SPE fever produc-
required prostaglandin synthesis, proba-
PGE_1 or PGE_2, and that the mechanism
ver production by SPE may be different
ı the mechanism underlying the enhance-
t of lethal endotoxin shock. Studies are
ently being done to elucidate further the
hanisms of fever production and en-
ement of lethal endotoxin shock by SPE.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 476–480 (1978)

Inhibition of Oxytocin Release by Morphine and Its Analogs (40080)

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Nutt and Jasinski (1) observed that administration of the morphine analog, oxilorphan, produced hypotonic polyuria in a group of heroin addicts. More recently Miller (2) reported that oxilorphan has a similar effect in Brattleboro rats heterozygous for the diabetes insipidus trait. He showed further that butorphanol, another morphine analog, was even more potent in causing polyuria in these rats. Both these investigators concluded that the analogs caused polyuria by inhibiting the release of the antidiuretic hormone (ADH) from the pituitary gland. Although it is well established that ADH and oxytocin (OT) can be released independently (3, 4) of each other, the cells producing these hormones are anatomically similar and closely associated. We thus thought it would be of interest to investigate how these two morphine analogs might affect release of OT in response to the physiological stimulus of suckling.

Materials and methods. All experiments were performed on Swiss Webster mice between the 11th and 22nd day of lactation. After the babies were born they were kept undisturbed for 10 days. On day 11 of lactation mothers were separated at about 10:00 AM and were reunited with their babies 4 hr later. They were then allowed to nurse their young for 1 hr. Babies were weighed just before and at the end of this hour of suckling. The difference of weight between the first and second weighings was assumed to be due to milk yield and was designated as "initial milk yield". All mothers then received an ip injection of 0.25 U of OT and were put back with their babies for another ½ hr of nursing. Babies were weighed again and the difference of weight between the second and third weighings was designated as the "residual milk yield". Babies were left with their mother overnight and separated the following morning. The same schedule was followed every day. On day 1 of experimentation mothers showed restlessness for about half an hour and they did not always provide a good initial milk yield. From day 2 on they seemed to be better adjusted to the experimental manipulations. Thus we always discarded the first day's results and the reported experiments were continued from day 2 for 10 days. In these experiments we did not cut down litter size to equal numbers but, instead, expressed the milk yield as g/100 g body weight of the litter.

Animals were divided in groups according to the design of the experiment. To avoid repetition of drug administration for two consecutive days to the same mice we altered the sequence of injection, i.e. the group which served as control on day 1 received butorphanol the next day and the group which received butorphanol on day 1 received oxilorphan the next day and so on.

Butorphanol, oxilorphan and morphine were always freshly diluted in isotonic saline and usually injected subcutaneously (s.c.) to the mother immediately before the 1 hr suckling period. In one set of experiments with butorphanol the drug was injected at different times to determine the best time for producing maximum inhibition. Control animals were injected with saline. Naloxone solutions were also made freshly every day and injected s.c. immediately before the 1 hr suckling period. An exception was the group which received both naloxone and morphine, where naloxone was given 10 min before morphine. Oxytocin, 0.25 U was injected intraperitoneally at the end of the 1 hr suckling period.

Butorphanol and oxilorphan are two newly synthesized morphine analogs. Their structures are given in Fig. 1. For comparison, structures of morphine and naloxone are also given. Naloxone, butorphanol and oxilorphan differ from morphine mainly by possessing a hydroxyl group at the 14 position. Butorphanol and oxilorphan can be fully synthesized whereas naloxone must be derived from thebaine, a natural alkaloid, as starting material.

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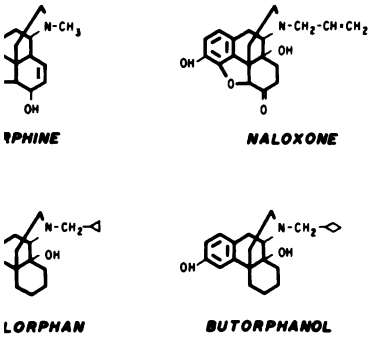
ıhanol and oxilorphan were gifts istol Laboratories, Syracuse, N.Y. ɛ was a gift from Endo Lab, Inc., City, N.Y. Oxytocin (Syntocinon) ift from Sandoz Pharmaceuticals. e sulphate was purchased from ıd Co., Inc.

:. Figure 2 illustrates the effects of nol and oxilorphan on initial milk th butorphanol and oxilorphan in- ıitial milk yield although the former to be a much more potent inhibitor. termine whether the reduced milk the presence of butorphanol and ın might reflect a reduction of milk ammary gland, 0.25 U OT was in- traperitoneally in all mice, treated rol, after the initial 1 hr milk yield Table I shows the total milk yield, ıl plus residual. Total milk yield in nol and oxilorphan-treated mice ɛss and actually a little more than in ıice. Thus the reduced milk yield in ɛnce of the drugs is not due to a ı of milk in the gland but is more e to inhibition of milk ejection me- OT.

l out the time of injection needed to ıaximum inhibition of initial milk injected 10 mg/kg doses of butor-



PHINE NALOXONE

LORPHAN BUTORPHANOL

Structures of oxilorphan and butorphanol with those of morphine and naloxone.

phanol at 0, 30, 60 and 120 min before the suckling period. The result is illustrated in Fig. 3. Maximum inhibition of 85% was obtained when butorphanol was injected immediately before suckling. Little more than 50% inhibition was observed when the drug was injected 30 min before and only 20% inhibition when given 120 min before the onset of suckling.

Pharmacological studies on oxilorphan and butorphanol clearly demonstrate that both drugs possess a mixture of analgesic and antagonistic properties (5, 6). To determine whether the analgesic or the antagonistic property of these drugs was responsible for the inhibition of OT release, we tested the effects of morphine, a purer agonist, and of naloxone, a purer antagonist, on OT release. The results of such experiments are given in Fig. 4. Naloxone at a dose of 5 mg and 10 mg/kg did not inhibit initial milk yield. However almost 95% inhibition was observed when mice were given 10 mg/kg of morphine. Thus morphine inhibited initial milk yield as much as or even more than butorphanol. If this inhibitory effect of morphine was a spe-

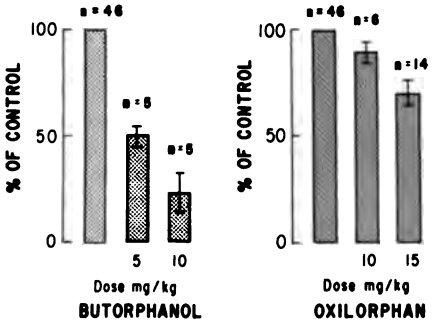


FIG. 2. Effects of butorphanol and oxilorphan on initial milk yield. Initial milk yield was calculated as described in Methods. Butrophanol and oxilorphan were injected subcutaneously immediately before the 1 hr suckling period. One hundred percent milk yield of control ranged from 1.14–5.03 g with a mean of 2.92 ± 0.15.

TABLE I. EFFECTS OF BUTORPHANOL AND OXILORPHAN ON TOTAL MILK YIELD.

	Total milk yield (g/100 g body weight)		
ıl (10)[a]	2.40 ± 0.29[b]	control (12)	3.45 ± 0.50
ıhanol (6) 5 mg/kg	2.88 ± 0.46	oxilorphan (5) 10 mg/kg	3.88 ± 0.68
ıhanol (6) 10 mg/kg	2.94 ± 0.44	oxilorphan (14) 15 mg	4.35 ± 0.69

r of animals is given in parenthesis.
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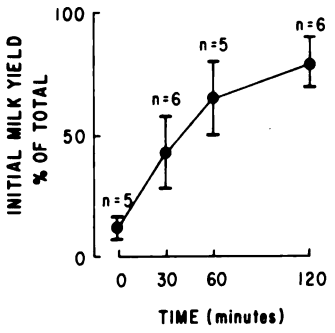

FIG. 3. Effect of time of injection of butorphanol on inhibition of initial milk yield. Butorphanol was injected at 0, 30, 60 and 120 min before the 1 hr suckling period. Initial milk yield is expressed as percentage of total. Dose of butorphanol, 10 mg/kg body weight.

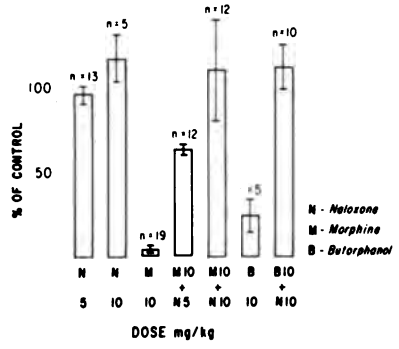

FIG. 4. Inhibitory effects of morphine and butorphanol on initial milk yield and their reversal by naloxone. In groups which received either naloxone or morphine, injection was made immediately before suckling. In groups which received both naloxone and morphine or butorphanol, naloxone was injected 10-min prior to morphine or butorphanol. The capital letters on the abscissa represent drugs and the numbers represent dose of the drug in mg/kg body weight.

cific effect of the narcotic it should be prevented by naloxone. As can be seen in Fig. 4, naloxone in a dose of 5 mg/kg partially prevented the inhibitory effect of morphine and a complete reversal of morphine inhibition was achieved by a dose of 10 mg/kg. Figure 4 illustrates further that the higher dose of naloxone effectively reversed butorphanol induced inhibition also.

Experiments were designed to determine whether the inhibition of milk yield in response to morphine was due to central inhibition of OT release or due to unresponsiveness of the mammary gland to endogenous OT. The response to intravenous injections of OT was tested in anesthetized lactating rats in a set-up similar to that described by Bisset *et al.* (7). Oxytocin was injected before and after subcutaneous administration of morphine at a dose of 10 mg/kg. Figure 5 illustrates the result of one such experiment. Oxytocin was administered in a dose of 0.1 mU, 1 min after and at 5-min intervals up to 75 min following morphine administration. The magnitude of milk ejection responses due to OT were not altered by preinjection of morphine.

Discussion. The results presented in this paper clearly demonstrate that both butorphanol and oxilorphan inhibit initial milk yield in lactating mice. They also show that butorphanol is a stronger inhibitor than oxilorphan.

The finding that babies were able to get a considerable amount of milk following OT injection indicates that a lack of circulating OT was probably responsible for the reduced milk yield in the drug treated animals. Thus the inhibition of milk yield was apparently due to inhibition of OT release into the blood and not due to unresponsiveness of the mammary gland to endogenous OT. Results obtained with lactating rats in which morphine failed to alter oxytocin-induced milk ejection fit nicely with this view.

The pharmacological studies of Pircio and Glyss (5) on oxilorphan and of Pircio et al. (6) on butorphanol show that both of these compounds possess a mixture of agonistic and antagonistic activities. Butorphanol is a stronger analgesic while oxilorphan is a stronger antagonist. Our finding that mor-

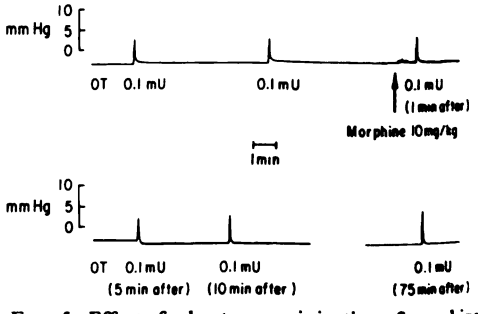

FIG. 5. Effect of subcutaneous injection of morphine, 10 mg/kg, on milk ejection response obtained by 0.1 mU OT injected intravenously. Following morphine injection OT was administered 1 min after and at every 5 min until 75 min.

ɔut not naloxone, was a potent inhib-
OT release explains why butorphanol
tronger inhibitor than oxilorphan.
results presented in this paper, how-
ɔ not shed any light on how oxytocin
is inhibited by the agonist drugs. We
rom the classical work of Douglas (8)
Douglas and Poisner (9) with isolated
y glands that Ca^{2+} is extremely im-
for the release of posterior pituitary
ıes. Thus a certain amount of Ca^{2+}
e present in order to have successful
ıe release. Although morphine has
ported to lower Ca^{2+} in different re-
f brain, particularly in the hypothala-
0), it is unlikely that the local Ca^{2+}
vould ever be low enough to interfere
T release.
s been reported by several investiga-
1–15) that morphine releases ADH.
er, the mechanism of such release is
ar. According to Schmidt and Living-
5), the antidiuretic action of morphine
ast partly due to its cardiovascular
On the other hand, morphine has
ported to release catecholamines from
ɔothalamus both in rats (17) and mice
nce there are several lines of evidence
techolamines cause release of ADH
) it is possible that the release of ADH
phine is an indirect one via the release
cholamines.
s (21), in his elegant experiments on
, showed how catecholamines can
)T release centrally. Activation of the
heticoadrenal system causing release
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ɔ block OT release. More recently he
l that norepinephrine causes inhibition
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ell bodies of the paraventricular nuclei
hus it is possible, in the experiments
d here, that the observed inhibition of
ease by morphine is due to the release
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tever may be the exact mechanism of
on it is certain that the inhibitory ef-
morphine and butorphanol are spe-
ıes since they are completely blocked
njection of a proper amount of nalox-
may be noted that the dose of nalox-
., 10 mg/kg, needed to achieve com-
lockade of morphine inhibition is sig-
nificantly higher than the blocking dose reported by Markowitz *et al.* (23). In their experiment 0.1 mg/kg naloxone could block the analgesic effect of 10 mg/kg morphine. However, it is hard to compare analgesic effects of morphine to its other effects since varying amounts of naloxone are needed to block analgesia when this is assessed by different methods.

This paper presents clear evidence of a new effect of morphine which was hitherto unknown. Inhibition of oxytocin release by morphine raises the questions whether endorphins, which have morphine-like properties, will also inhibit OT release and whether endorphins may participate in the physiological regulation of OT release.

Summary. Two synthetic morphine analogs, butorphanol and oxilorphan have been found to inhibit suckling induced oxytocin (OT) release in lactating mice. Morphine when tested in a similar experimental set-up produced even greater inhibition. Naloxone could effectively block inhibitions induced both by butorphanol and by morphine. When injected alone, naloxone did not produce any inhibition. These results indicate that (a) butorphanol, oxilorphan and morphine inhibit suckling induced OT release in mice, (b) the analgesic property of these compounds is responsible for the inhibition, and (c) inhibition produced by morphine and butorphanol is specific in nature since it could be reversed by naloxone.

The authors wish to thank Becky Wo for her excellent technical assistance. Thanks are also extended to Dr. Frank S. Caruso of Bristol Laboratories for a generous supply of butorphanol and oxilorphan. This work was supported by Research Grant No. AM 01940 from the NIAMDD.

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The Kinetics of Serum Ferritin[1] (40081)

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:rritin is widely distributed in body tis- where it constitutes the major form of ıge iron. It was originally believed that tin is normally confined to intracellular partments. However, it has been shown ıtly that minute amounts of ferritin can riably be detected in human sera by sen- : immunologic measurements and that level of the serum ferritin provides an rate measure of iron stores (1–5). Com- d with the rapid accumulation of data rding the clinical significance of the se- ferritin, the physiology of this parameter orly understood. The reticuloendothelial :m located in the liver, spleen, and bone ·ow (RES) appears to be the major pre- ɔr compartment based on radioactive la- ng measurements in the rat (6) and clin- correlations in man (5). Relatively large s of recrystallized ferritin in rats are rap- cleared by the hepatic parenchymal cell nd a rapid clearance has also been dem- rated in man although the major site of ke was not identified (8). In the present y, a dog model was employed to define kinetics of serum ferritin. The effect on tin clearance of the amount of material :ted, its iron content, and its method of fication was studied.

aterials and methods. Studies were per- ıed in adult mongrel dogs of either sex hing between 13 and 19 kg. All animals : dewormed and kept under observation t least 14 days prior to study. The animals : housed in individual cages and fed reg- dog chow. To examine the effect of iron .s on ferritin clearance, two animals were e iron deficient as determined by bone ·ow examination after removing 450 ml ood 7 and 14 days prior to study.

ɔr kinetic measurements, animals were thetized with iv phenobarbital. One ml blood was drawn prior to and at frequent intervals for at least 30 min following the injection of ferritin. The rate of clearance was determined by least squares regression, assuming exponential clearance, and the recovery of injected ferritin was calculated from the extrapolated time zero value and an estimated plasma volume of 35 ml/kg.

Recrystallized ferritin was prepared from dog liver by the method of Mazur and Shorr (9). Five times recrystallized ferritin was stored at 4° as an $(NH_4)_2SO_4$ precipitate. Prior to use, aliquots were redissolved in 0.2 *M* borate buffer and extensively dialyzed against the same buffer. The final preparation was chromatographed on Sephadex G200 (Pharmacia, Upsula, Sweden). The concentration of ferritin was measured by Lowry's method (10) using a bovine serum albumin standard.

To prepare apoferritin, recrystallized ferritin was dialyzed overnight against 0.1 *M* acetate buffer, pH 5.2, and for a further 90 min against the same buffer containing 0.1 *M* thioglycollic acid. The colorless solution was then dialyzed against repeated changes of 0.05 *M* acetate buffer, pH 5.2, followed by deionized water and finally 0.2 *M* borate, pH 8.0 containing 0.15 *M* NaCl (borate buffer).

Recrystallized ferritin was tagged with ^{59}Fe by incubating 2 mg ferritin in 1 ml borate buffer with 150 μCi ^{59}Fe in 0.1 ml 1 *N* HCl containing 1 *M* freshly prepared ascorbic acid. The mixture was incubated at 4° for 2 hr with continuous oxygen agitation. Unbound ^{59}Fe was removed by 18 hr dialysis against 0.1 *M* EDTA followed by 24 hr dialysis against borate buffer. At that time 29% of the original ^{59}Fe was bound to ferritin and of this, 98% was precipitated by antiferritin antiserum. Recrystallized ferritin was iodinated with ^{125}I by the method of Hunter and Greenwood (11). Unbound radioiodine was removed chromatographically with Sephadex G25. Studies were also performed with crude (uncrystallized) ferritin prepared from dog

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liver and spleen. The latter tissues were homogenized in 4 vol cold 1 *N* NaCl, ultrasonicated for 1 min, heated to 70° for 10 min, and centrifuged at 25,000*g* for 1 hr at 4°.

Serum ferritin levels were measured by immunoradiometric assay (IRMA) as described by Miles *et al.* (12). The characteristics of this assay were similar to those previously reported for human serum ferritin measurements. In 19 normal animals the geometric mean serum ferritin was 24 ng/ml with a range from 7 to 75 ng/ml (± 1 SD 12–47 ng/ml).

Results. The clearance of recrystallized dog ferritin in doses greater than 500 μg was determined by changes in the serum ferritin level as measured by IRMA. A typical clearance curve with a dose of 5.2 mg recrystallized ferritin is shown in Fig. 1. The injected ferritin was cleared with a half time ($T_{1/2}$) of 6.6 min with a recovery of 101%. Following the exponential decline of 80–90% of the injected ferritin, the rate of clearance slowed considerably, presumably due to a return to the plasma compartment of a portion of the ferritin cleared initially. In all remaining studies clearance rates were determined from the initial linear segment of the curve.

Effect of dose. Since the level of serum ferritin in various clinical disorders ranges from 1 to several 1000 ng/ml, the effect on clearance of the amount of injected ferritin was examined. With doses greater than 400 μg, clearance could be determined from changes in the serum ferritin level as measured by IRMA. The results of studies performed with doses between 400 and 5,200 μg recrystallized ferritin are summarized in Table I. Clearance $T_{1/2}$ was uniformly less than 10 min, ranging from 5.8 to 8.7 with a mean of 7.1 min. There was no apparent relationship between the rate of clearance and either the quantity of injected ferritin or the basal serum ferritin level of the dog.

Recrystallized ferritin labelled with ^{125}I was used to study the clearance of a smaller amount of injected ferritin. With 3 μg of labeled ferritin, the clearance $T_{1/2}$ was 7.3 min, identical to the mean $T_{1/2}$ obtained with doses 100–1000 fold higher. In a final study this same dose of ^{125}I labelled recrystallized ferritin was mixed with 3000 μg unlabelled carrier ferritin prior to injection. A rapid $T_{1/2}$ of 6.9 min indicated no effect of this 1000-fold increase in injected dose.

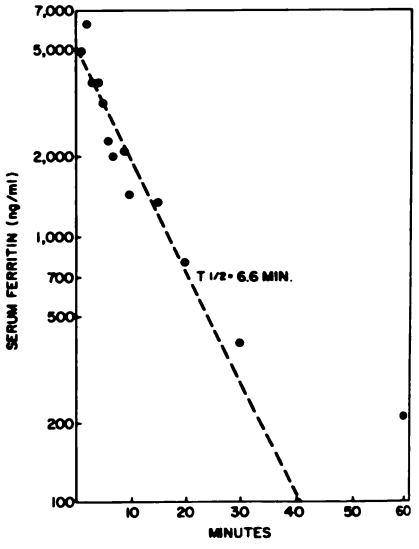


FIG. 1. The clearance of 5200 μg recrystallized ferritin. The interrupted line was determined by least square regression.

Ferritin iron clearance. A study was performed to determine whether iron contained in ferritin is cleared at the same rate as the protein moiety. Ferritin tagged *in vitro* with ^{59}Fe was injected in an amount sufficient to permit measurement of protein clearance by IRMA simultaneously. In the first study performed with 1200 μg recrystallized ferritin the clearance $T_{1/2}$ for ^{59}Fe was 8.3 min as compared with 7.1 min for the ferritin protein (Fig. 2). The difference was not statistically significant. In a second study performed with 700 μg ferritin, similar clearance $T_{1/2}$ values of 7.9 and 7.6 min were observed for ^{59}Fe and ferritin protein respectively. In both these studies the residual plasma ^{59}Fe could be preciptated with an excess of antiferritin antiserum.

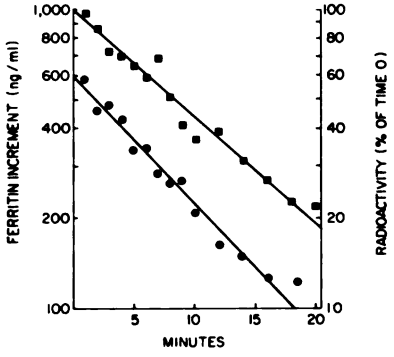
Effect of iron content. To determine the iron content of ferritin influences the clearance rate of the protein, the clearance of recrystallized ferritin was measured following complete removal of iron to obtain apoferritin. In two separate studies performed with 1000 and 1200 μg ferritin, the clearance $T_{1/2}$ increased significantly to 25 and 16 min respectively (Fig. 3A).

Effect of purification procedures. To deter-

ABLE I. THE RATE OF CLEARANCE OF RECRYSTALLIZED HEPATIC FERRITIN AS MEASURED BY IRMA.

ly No.	Dog wt.	Dose of ferritin	Preinjection serum ferritin	Serum ferritin increment	$T_{1/2}$	Recovery[a]
	(*kg*)	(*μg*)	(*ng/ml*)	(*ng/ml*)	(*min*)	(%)
1	26	5200	114	5808	6.6	101
2	16	3200	118	8818	5.8	154
3	25	700	20	625	7.0	78
4	26	650	120	501	7.4	70
5	16	400	180	975	8.7	136

ased on a plasma volume of 35 ml/kg.



G. 2. The clearance of 1200 μg recrystallized fer-agged with ^{59}Fe. Clearance $T_{1/2}$ of the protein as ured by IRMA (●) was 8.3 min and the clearance e (■) was 7.1 min.

e the possible effect of recrystallization erritin clearance, studies were performed relatively crude ferritin recovered in the ed supernatant of dog liver or spleen ogenates. In two separate studies with liver ferritin, the clearance $T_{1/2}$ of 3010 2930 μg ferritin as determined by IRMA 35 and 18 min respectively (Fig. 3B). ilar results were obtained with 1850 μg 1300 μg ferritin from dog spleen; the rance $T_{1/2}$ was 32 and 33 min respectively . 3C).

ffect of iron status. The serum ferritin l is roughly proportional to iron stores in although it has not yet been determined ther these changes are due to differences ie entry or in the clearance of circulating tin. The latter possibility was examined neasuring the clearance of recrystallized tin in phlebotomized dogs. In two studies clearance $T_{1/2}$ of 1200 μg recrystallized tin was 5.8 and 9 min which are similar ilues obtained in iron replete animals.

iscussion. It has not been possible to di-y measure the influx of serum ferritin in human subjects. However, there is circumstantial evidence that the RES is the immediate precursor of the circulating protein. For example, the sudden reduction in RES stores of iron which occurs following phlebotomy is reflected by an equally precipitous drop in serum ferritin. In a wide spectrum of clinical disorders there is an excellent correlation between serum ferritin and RES stores as estimated directly by histologic grading of a bone marrow aspirate (5). The sudden block in transferrin loading of iron by RES which occurs with induced fever or elective surgery produces an appropriate reciprocal rise in the serum ferritin (13). In patients with on-going infection or inflammation, the serum ferritin may be disproportionately elevated in relation to marrow iron stores although the correlation still holds (5). More direct evidence that the RES accounts for the major influx of ferritin to serum was obtained in rats by Siimes and coworkers (6). Following the injection of ^{59}Fe tagged red cells damaged by heating to ensure prompt removal by RES, radioactivity promptly appeared in circulating ferritin within 20–40 min. In contrast, no labelling of ferritin was observed following the administration of oral radioiron or ^{59}Fe hemoglobin–haptoglobin which is removed by the hepatic parenchymal cell (14). Thus, while other tissues might well contribute to circulating ferritin, the RES seems to be the major source.

Data on the clearance rate and sites of tissue uptake of circulating ferritin is also limited. It has been shown in rats that recrystallized ferritin tagged with ^{59}Fe is removed almost exclusively by the hepatic parenchymal cell (7). Relatively large doses above 200 μg are removed with $T_{1/2}$ of 30–40 min (15), whereas smaller doses below 1 μg are removed more rapidly with a half time of less

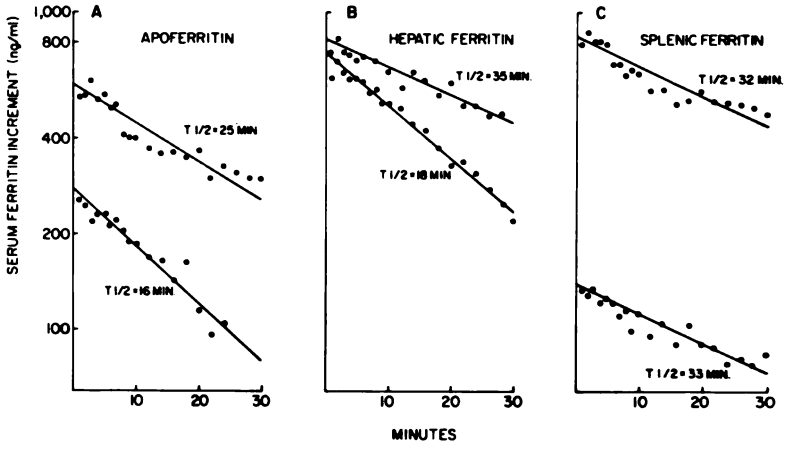


FIG. 3. The clearance of apoferritin (A), (crude noncrystallized) hepatic (B) and splenic (C) ferritin. The result of two separate studies are shown for each preparation. In all cases clearance was significantly prolonged as compared with recrystallized ferritin.

than 5 min (6). The only observation on the clearance rates of serum ferritin in man was obtained in newborn infants requiring exchange transfusions for hyperbilirubinemia (8). Based on the difference between the plasma ferritin of the transfused blood and in the infant both before and following blood transfusion, a very rapid $T_{1/2}$ between 2.5 and 5.5 min was observed in two infants with birth weights above 2000 g. In the infants with lower birth weights, below 1200 g, slower clearances of 9.1 and 34 min were seen. Because of this very rapid turnover of serum ferritin, it has been calculated that if the circulating protein has a normal iron content of 20–25%, the amount of iron transported through the serum as ferritin each day could equal the daily plasma iron turnover from the breakdown of senescent red cells.

None of the observations reported here are inconsistent with published data on serum ferritin kinetics. In the present study the clearance $T_{1/2}$ was invariably less than 10 min with doses ranging from 3 μg protein to 5.2 mg. This would suggest that in this animal model clearance of ferritin is not rate limiting and that any changes in the circulating level reflect differences in the rate of entry rather than the rate of removal from the circulation.

It was also observed in this study that ferritin iron is not selectively removed from the circulating protein but is cleared simultaneously. Moreover, because the serum radioactivity following ^{59}Fe ferritin injection was precipitable with excess antiferritin antiserum, there is not direct transfer of iron from ferritin to transferrin. However, it was observed that removal of iron from recrystallized ferritin resulted in a substantially slower clearance of the protein. This finding is of interest in light of recent studies in man which suggest that the circulating protein contains no iron (16). Serum ferritin turnover may be substantially slower than that observed with recrystallized ferritin in these studies and there may be no appreciable transport of iron through this compartment. Evidence was also obtained in this study that the method of purifying ferritin may significantly influence its rate of clearance. It is now known that both serum and tissue ferritin actually consist of a family of isoferritins which can be identified by isoelectric focusing (17, 18). Furthermore, studies in man indicate that recrystallization selects out predominantly basic isoferritins which are less heterogeneous than the mixture of isoferritins obtained without recrystallization (19). This may explain why recrystallized ferritin is cleared more rapidly than the ferritin recovered from tissue without crystallization. Obviously, any final interpretation of the kinetic data observed in this model must await more detailed characterization of the circulating protein.

Summary. The mean serum ferritin in normal dogs averaged 24 ng/ml which is similar to the level in normal man. The plasma clearance of recrystallized ferritin was determined

by changes in the serum level or by the clearance of ferritin tagged with ^{125}I or ^{59}Fe. Clearance half times was invariably less than 10 min with doses ranging from tracer quantities to several mg protein. The iron and protein moieties of ferritin were removed simultaneously, but clearance of the protein was slower when iron was removed before injection. The clearances of relatively crude hepatic or splenic ferritin preparations were also slower, suggesting that isoferritins may differ substantially in their rate of clearance.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 486–488 (1978)

Release of Tissue Taurine from the Oxygen-Deficient Perfused Rat Heart (40082)

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Taurine, 2-aminoethanesulfonic acid, is present in high concentrations in cardiac muscle (1). The function of this naturally-occurring substance has not been defined, although it has been implicated in maintenance of normal electrophysiological function of the heart. For example, it has been reported that taurine is capable of reversing electrocardiographic abnormalities induced by high concentrations of certain glycosides (i.e. digoxin and strophanthin K) (2, 3). In this context, taurine may be involved in regulating ion fluxes, specifically K^+ and Ca^{2+} (4, 5).

We have recently reported both a regional heterogeneity in taurine levels within the dog myocardium and a generalized decrease in the taurine content in ischemic cardiac muscle (6). The ischemic left ventricle, induced by coronary artery ligation, demonstrated a loss in taurine content exceeding 45%. The loss of taurine in the myocardium could have occurred by at least two mechanisms: (a) metabolism or (b) release into the general circulation. While it has been demonstrated in tracer studies that taurine is indeed metabolized to isethionic acid, the rate is extremely slow (7, 8). Therefore, in quantitative terms the loss of taurine during short periods of ischemia (4 hr) due to metabolism could be eliminated as a possible mechanism. Since taurine is normally present in blood, albeit in low concentration, and since ischemic damage is principally a manifestation of cellular hypoxia, we have attempted to reproduce taurine loss in an isolated heart perfused with a taurine-free and oxygen-deficient buffer.

The present report shows, indeed, that myocardial cellular hypoxia resulted in a loss of tissue taurine *per se*. This taurine was recovered quantitatively in the buffered perfusate.

Materials and methods. Animals. Male Sprague–Dawley rats weighing 250–300 g were given free access to Purina rat chow and water.

Perfusion system. The animals were anesthetized with ether, the chest opened, heart excised and mounted on the perfusion apparatus as described elsewhere (9–11). Coronary perfusion pressure was constant at 82 mmHg. One group of hearts was perfusion-washed in a nonrecirculated system for 5 min with Krebs–Henseleit bicarbonate buffer containing 5.5 m*M* glucose. The ventricles were freeze-clamped with Wollenberger tongs cooled in liquid nitrogen and the frozen tissue analyzed for "initial" taurine content. A second group of hearts was perfused for an additional 30 min ("residual") before freeze-clamping and analysis. Each of the groups mentioned above was further divided into two groups; in one group, the buffered perfusate was equilibrated with 95% O_2–5% CO_2 (oxygenated controls) and in the other, the medium was gassed with 95% N_2–5% CO_2 (oxygen-deficient). The perfusion medium was sampled at 10-, 20- and 30-min intervals and aliquots taken for taurine analyses. Mean coronary flow rates (ml/min/g wet wt ± SE) were 10.9 ± 0.8 and 16.8 ± 1.0 for oxygenated controls and oxygen-deficient hearts, respectively. Mean heart rates (beats/min ± SE) were 286.6 ± 24.0 for oxygenated hearts and 130.0 ± 38.7 for oxygen-deficient hearts.

Estimation of tissue and perfusate taurine. The cardiac tissue was weighed in the frozen state and then homogenized in 3 vol of cold 2% perchloric acid with a Virtis Model 45 homogenizer for 20 sec at half speed. The homogenate was centrifuged for 10 min at 12,000*g*. Aliquots of the supernatant were analyzed for taurine content by an amino analyzer procedure as described previously (12).

A portion of ventricle from each heart was dried to constant weight and the dry: wet ratio determined. All data are expressed on a dry weight basis. A programmed statistical analysis of the data was performed on an IBM 370/145 computer.

Results. The effects of oxygen deficiency

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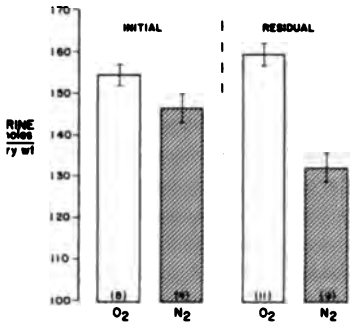

Taurine content in rat heart after perfusion ithout oxygen. Hearts were perfused with ıselcit bicarbonate buffer containing 5.5 m*M* · 5 min ("initial") or for an additional 30 min '). Clear bars and hatched bars show values taurine in hearts perfused with buffer equili- h 95% O_2–5% CO_2 and 95% N_2–5% CO_2, y. Each value represents the mean ± SE of the hearts indicated in parenthesis.

taurine content are shown in Fig. 1. hown on the left of the figure repre- rine content after a nonrecirculated m washout of the coronary vascular thus reflect a more accurate estima- initial tissue taurine. The apparent e between values from normoxic and erfused hearts was not statistically nt ($P > 0.10$). Comparison of "ini- l "residual" values showed that tissue levels remained unchanged after 30 ell-oxygenated perfusion ($P > 0.10$). r, a similar period of perfusion under onditions resulted in a decrease in of taurine ($P < 0.02$).

taurine was not present in the per- edium initially, samples of perfusates lected at various time intervals during ted perfusion. The results are shown . In well-oxygenated control hearts, uantity of taurine was detected after tes of perfusion. In this group of erfusate taurine amounted to less of initial tissue values which was methodological error. No further f taurine was observed after the ini- in sampling. In hearts perfused with ree medium, taurine release in- three- to fourfold after 10 min of ted perfusion and continued to in- rough 30 min. Comparison of values e taurine loss and those for appear- taurine in the perfusion medium of oxygen-deficient hearts after 30 minutes indicated approximately 100% recovery.

Discussion. The role of taurine in tissue function is for the most part uncertain; the exception being its known conjugation with specific bile acids (13). In recent years, however, evidence has accumulated to suggest a functional role of taurine in heart; a tissue which contains a relatively high content of this sulfonic amino acid. Observations by Kocsis *et al.* (14) and Crass and Lombardini (6) have shown that dog myocardial tissue displays a regional heterogeneity in taurine content, including an increasing outer to inner transmural gradient across the left ventricular wall.

A potentially important function of taurine in heart was suggested in the experiments of Read and Welty (2) and others (3). These workers noted that infusion of taurine into animals demonstrating glycoside-induced electrocardiographic abnormalities resulted in the disappearance of the arrhythmias. These and other more recent observations which have recently been reviewed (15) have implicated taurine, or its principal metabolite, isethionic acid, in regulation of intracellular K^+ and Ca^{2+} fluxes. In this context, we recently postulated (6) that a contributing factor to the arrhythmias characteristic of acute myocardial ischemia could be the loss of intracellular taurine. The cardiac muscle cell maintains a large taurine gradient relative to the extracellular fluid (3). A decrease in cellular phosphate potential secondary to severe

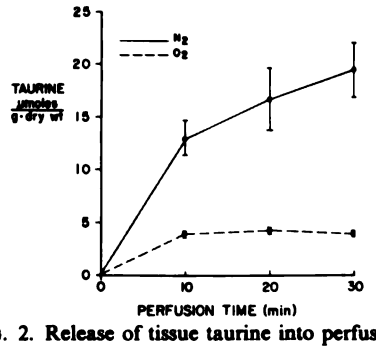

FIG. 2. Release of tissue taurine into perfusate during anoxic perfusion of the isolated rat heart. Hearts were perfused with Krebs–Henseleit bicarbonate buffer containing 5.5 m*M* glucose equilibrated with either 95% N_2–5% CO_2 (solid line) or 95% O_2–5% CO_2 (broken line). Each value represents the mean cumulative taurine value in the recirculated perfusate of six hearts at the times indicated ± SE.

hypoxia may, as in the case of K^+ loss (16), be the basis of taurine loss observed in ischemic heart muscle (6). Taurine loss is particularly large in the subendocardial region where density of conduction tissue is greatest (14, 17).

In the present studies, isolated rat hearts, deprived of oxygen to simulate ischemia-induced cellular hypoxia, were perfused with a taurine-free medium to facilitate detection of possible tissue taurine leakage. In hearts perfused with oxygen for 30 min, the tissue content of taurine was not significantly changed (Fig. 1). However, within 10 minutes the perfusate contained 3.88 μmoles of taurine/g dry wt (Fig. 2). The recovery of taurine in the oxygenated perfusate was due, perhaps, to the short period of hypoxia (<1 min) when the heart was removed from the animal and mounted on the perfusion apparatus. Furthermore, the observation that the taurine release into the medium did not increase with time tended to support this conclusion.

In hearts perfused with the nitrogen mixture, a significant decrease in tissue taurine content was observed (Fig. 1). The "lost" taurine was recovered 100% in the perfusate (Fig. 2). Moreover, the release of taurine under conditions of oxygen deficiency increased with time (Fig. 2).

The data obtained in these experiments demonstrate that whole heart hypoxia, as in the case of acute ischemia *in vivo* (6), results in taurine loss. Furthermore, loss of taurine appears to involve leakage of taurine *per se* as opposed to hypoxia-induced metabolic conversion to a taurine metabolite. The decrement in the tissue pool(s) of taurine was recovered quantitatively in the perfusion fluid.

It would be of interest to determine whether a nonrecirculated perfusion system involving continuous removal of released taurine would potentiate tissue loss; and conversely, whether supplementation of the perfusion medium with exogenous taurine in anoxic or ischemic hearts could prevent tissue taurine leakage. Experiments to test these questions as well as efforts to correlate severity of electrophysiologic abnormalities with extent of taurine loss are currently in progress.

Summary. The effects of cellular hypoxia on taurine levels in rat hearts were determined. Hearts perfused with 95% N_2–5% CO demonstrated a significant decrease in tissu taurine content when compared to contro hearts perfused with 95% O_2–5% CO_2. Th loss of taurine in oxygen-deficient hearts wa time dependent over a period of 30 min. Th perfusate when analyzed for taurine conten contained 100% of the released taurine. Thus metabolic conversion of taurine had no rol in the disappearance of taurine from the ra heart.

The authors thank Mr. Harvey Olney of the Depart ment of Biochemistry, Texas Tech University School o Medicine, for performing the taurine analyses by th amino acid analyzer procedure. The studies presente here were supported in part by NIH research grant I R01-NS-11406 from the National Institute of Neurolog ical Diseases and Stroke and the Texas Affiliate of th American Heart Association.

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ties of Epstein-Barr Virus Transformed Woolly Monkey Lymphoblastoid Cell Lines (40083)

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>rld primates are useful experimen-
tory models for Epstein-Barr virus
fections (1). These species do not
iarbor EBV or a related virus in
t their cells are susceptible to trans-
in vitro (2–4). In limited studies
l so far lymphoma has been in-
otton-top marmosets (Saguinus) (3,
monkeys (Aotus) (6) and in squirrel
(Saimiri) (7). However, not all in-
of each species are susceptible to
esis or even to persistent infection.
ple, squirrel monkeys with malaria
fulminant lymphoproliferative dis-
inoculation with autochthonous
erted cells (7), but only transient
responses were found in normal
ionkeys (2). In some species, such
rix marmosets, only mild lympho-
ve disease has occurred after EBV
n (8).

/ariable responses of non-human
nay be explained by differences in
nts and types of virus or trans-
lls inoculated and also by host dif-
he nature of which remains to be

report we described the derivation
ous woolly monkey (WM) lympho-
nes following *in vitro* transforma-
ikocytes by an EBV strain (B95-8)
l previously been shown to be on-
the cotton-top marmoset (CTM).
WM cells differed from CTM cells
face markers, virus production and
ity. These differences serve as a
exploring factors which might ac-
the variations in pathogenic poten-
V in different hosts.

ls and methods. Cells. Mononuclear
ns were obtained from heparinized
blood utilizing Ficoll–Hypaque
9). Populations depleted of T lym-
were derived by a second Ficoll–Hypaque separation of cells which had formed rosettes with neuraminidase treated sheep erythrocytes (Vibrio cholera, Calbiochem). Lymphoblastoid lines were established following *in vitro* exposure of 2–8 × 10^5 cells to 10^4 transforming units of the B95-8 EBV strain (10).

Lymphocyte markers. Cells with receptors for C3 were identified by the "EAC" rosette test. The source of antibody was 19 S rabbit anti-sheep erythrocyte, and the source of complement was fresh mouse serum. Receptors for the Fc portion of IgG were sought with the "EA" rosette test. Two sets of reagents were employed: sheep rbc coated with a nonagglutinating dilution of 7 S rabbit anti-sheep rbc (Cordis Laboratories) and human type O Rh positive rbc coated with a 1:80 dilution of human anti-D γ globulin (Spectra Biologicals).

Cytogenetic and morphologic studies. Chromosome studies were performed with the Giemsa banding method (11) on metaphase cells derived from two immortalized woolly monkey cell lines as well as from freshly drawn peripheral blood leukocytes from an uninoculated monkey.

Tests for EBV in transformed cells. Lines were tested for EB nuclear antigen and viral capsid antigen using positive and negative human reference antisera (12, 13). Early antigen was sought following treatment with iododeoxyuridine (IUdR) and following superinfection with the P_3J-HR-1 EBV strain (14, 15). Cell profiles from several lines were examined for viral nucleocapsids by electron-microscopy.

Animal inoculations. Four monkeys (Lagothrix lagotricha) were inoculated with cell-free B95-8 virus with a titer of 10^5TD_{50}/ml. They received 1 ml intravenously, 1 ml intraperitoneally, and 0.5 ml applied to the posterior pharynx with a cotton swab. Two of the four monkeys received azathioprine (3

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mg/kg) and prednisolone (0.15 mg/kg) for 20 days. Four monkeys received injections of 4×10^7–1×10^8 autologous cells first transformed *in vitro* by B95-8. Two of the monkeys which received autologous cells had received cell-free virus 9 months previously, but had not developed persistent antibodies. They received two sets of inoculations of autologous cells each: one was given intrathecally via the cisterna magna, and the second inoculation, 6 months later, was given intraperitoneally and into the buccal mucosa. The intraperitoneal inoculations were accompanied by immunosuppressive chemotherapy, as outlined above. Two other monkeys which had not previously received inoculations of virus were given $7–9 \times 10^7$ cells into the peritoneal cavity and 10^7 cells in each buccal mucosal surface; one was immunosuppressed.

EBV antibody. Antibodies to VCA and EBNA were measured in sera taken biweekly from inoculated animals (16).

Results. Establishment of lymphoblastoid cell lines (Table I). Woolly monkey peripheral blood lymphocytes were relatively resistant to *in vitro* transformation by EBV. Transformation in the EBV system is defined as continued proliferation of lymphocytes *in vitro* following virus exposure. Cell lines were established from four animals; considering only those animals in which transformation was successful (four of eight) only 10% (14/145) of virus-exposed leukocyte cultures resulted in cell lines. No lines were derived without *in vitro* exposure to virus.

We were unsuccessful in our attempts to increase transformation frequency by exposure of the leukocytes to mitogens (PHA, conA, PWM, LPS). Similarly we could not increase frequency of transformation by exposing the cells to virus after several days in culture when many of the cells had entered DNA synthesis. Depletion of T lymphocytes also did not affect transformation frequency.

Karyotypes were prepared from two cell lines (monkeys B and O) and from a fresh peripheral blood sample of monkey R. Primary cells from monkey R had a constant chromosome number of $2n = 62$. Most cells from line O contained 62 chromosomes; however, these appeared to be pseudodiploid. Cells from line B had a basic chromosome number of 61 with at least two probable translocation chromosomes and other abnormalities.

TABLE I. ATTEMPTS TO ESTABLISH LYMPHOBLASTOID CELL LINES FROM WOOLLY MONKEY LEUKOCYTES.

Monkey B[a]	3/15[c]	0/15
Monkey O[a]	3/14	0/15
Monkey G	0/7	0/7
Monkey D	0/7	0/7
Monkey P	2/96	0/53
Monkey M	0/4	0/4
Monkey W	0/18	0/19
Monkey R	6/20	0/12
Total[c]	14/181	0/132

[a] Animals immunosuppressed with azathioprine an prednisolone.

[b] B95-8 strain of EBV, with 10^4–10^5 transforming uni per ml.

[c] No. cultures positive/no. attempted.

Expression of EBV in transformed cells. A low level of viral capsid antigen (0.1–1.0% o cells) was detected in four of five virus transformed lines. Supernatant fluids from these lines also contained transforming virus demonstrated by lymphoblastoid transformation of umbilical cord leukocytes with freeze-thawed and filtered materials. The transforming titer in all lines was 10^0–$10^{0.5}$ 50% transforming units per 0.1 ml. The amount of virus in the extracellular fluid was assayed by end-point dilution transformation of fresh human umbilical cord lymphocytes. Titers were calculated by the Reed Muench formula. Of five lines tested for the presence of EBNA all were positive, but the reaction was less intense in the WM lines than in the Raji positive control and was characterized by a fine, flecked pattern rather than a coarsely granular one.

We attempted to detect early antigen (EA) in two WM cell lines and to influence its production by additon of IUdR (60 μg/ml) or by superinfection with HR-1 virus (5×10^4 EA inducing units/ml as measured in Raji cells (14, 15). Both cell lines spontaneously produced low levels (1–3%) of EA since more cells stained with the EA + VCA + serum than with a serum which only contained anti-VCA. Addition of HR-1 virus did not increase the number of fluorescent cells and the level of EA expression was not enhanced by IUdR.

We demonstrated herpesvirus particles by electron microscopy in a thin sectioned cell

prepared from one cell line. These cells been exposed to iododeoxyuridine .) for 72 hr 2 weeks earlier. One area of ibris contained seven capsids; one cap- ıtained a core. No C-type particles were Freeze-thawed supernatant fluids from ıame cultures caused transformation of ı umbilical cord leukocytes.

ıphocyte markers on primary and EBV ırmed WM leukocytes (Table II, III). 50% of WM peripheral blood mono- r cells isolated by the Ficoll Hypaque que are T lymphocytes, i.e., make ro- with sheep rbc (E). Nearly 50% of the ı peripheral blood have a receptor for s identified by the EA rosette marker, e presumably B lymphocytes and mon- , or, possibly, activated T cells. Cells g the IgFc marker are about two to imes as prevalent in WM blood as in ı or cotton-top marmoset blood (17). ximately 20% of WM mononuclear ave the C3 receptor as identified by the osette test.

ept for loss of T lymphocytes, the sion of lymphocyte markers on the ırmed cells reflected the markers found mary cells. The majority of cells in the xpressed the IgFc receptor. Overnight ıtion of cells in the WM lines with antibody-coated erythrocytes at 4° l 90% EA rosettes. EA rosettes were rmed when human anti-D coated Rh- e type O human erythrocytes were s the reagent (Table III). About 20% of ı the WM lines exhibited the C3 recep- nce cloning studies have not yet been ned with these lines, it is not known whether the EA and EAC receptors are present on the same cell and whether all cells have the capacity to express the EAC receptor.

Spontaneous expression of the EA receptor on WM cells is unusual for cells transformed *in vitro* by EBV and we have not encountered this receptor on comparable immortalized human or marmoset cells (17). We wished to determine the relationship of the EA receptor to that which is induced by herpes simplex virus, since we considered the possibility that WM cells might carry a related virus. After infection with HSV type 1, several Burkitt lymphoma cell lines and a WM line were tested for receptors for human and rabbit IgFc. From 2 to 4% of cells in the herpes infected Burkitt lymphoma lines developed receptors for rabbit IgFc but not human IgFc. EBV transformed WM cells expressed receptors for IgFc of both species, and these receptors were not altered following HSV infection. About 0.1% of cells in all four lines formed infected centers after HSV infection.

Response of animals to inoculation of virus and autologous EBV transformed cells. All monkeys remained well following inoculation of cell free virus and autologous transformed cells. No alterations in peripheral blood count, or blood leukocyte morphology were noted. No lymphadenopathy or masses were detected.

Autopsies were performed on three animals 6–22 months after inoculation of autologous transformed cells. Two monkeys had been inoculated with such cells in the cisterna magna. Both gross anatomy and histology were normal. Cell lines could not be estab-

E II. EXPRESSION OF SURFACE RECEPTORS ON FRESH PERIPHERAL BLOOD LYMPHOCYTES OF WOOLLY MONKEYS AND ON WOOLLY MONKEY LYMPHOBLASTOID CELL LINES.

	Peripheral blood lymphocytes			Lymphoblastoid cell lines		
imal	% EAC[a]	% EA(7S)[b]	% E[c]	% EAC	% EA(7S)	% E
1 P	18	45	55	18	67	0
1 P	18	52	56	29	75	0
1 R	18	43	47	27	45	0
1 O	ND[d]	ND	ND	21	48	0
1 B	ND	ND	ND	34	42	0
1 M	27	47	55	ND	ND	ND

centage of cells which form rosettes with sheep erythrocytes coated with rabbit 19S antibody and mouse nent.

centage of cells which form rosettes with sheep erythrocytes sensitized with rabbit 7S immunoglobulin.

centage of cells which bind sheep erythrocytes at 4°.

) = Not done.

lished from various lymphoid tissues at autopsy, nor could EBNA be demonstrated in imprints of lymph nodes.

All animals lacked antibody to VCA initially. Low level anti-VCA, at a titer of 1:10, appeared in the sera of three of four animals inoculated with cell-free virus by weeks 4 and 5 following inoculation, but was no longer detected by week 9 after inoculation. Similarly, only low level transient anti-VCA responses were observed in animals given autologous transformed cells.

Many attempts were made to recover lymphoid cell lines from the peripheral blood of inoculated animals at various intervals after inoculation. This was unsuccessful, with one exception: cell lines were established from several cultures of blood leukocytes obtained one week after intracisternal inoculation of autologous cells in one monkey. These lines presumably represent transformed cells which found their way to the periphery and circulated transiently.

Discussion. We report these findings for two reasons. First, the surface properties of EBV-transformed WM lymphocytes are unusual. The lines express avid IgFc receptors as demonstrated by the EA rosette test and thus they may be useful in further work on this receptor. Second, although the number of inoculated animals is small, WM cell lines immortalized in vitro by EBV have not proved oncogenic when returned to the autologous host, even in an immunologically privileged site or when administered with immunosuppressive drugs.

The IgFc receptor found on transformed WM cells is not found on comparable human or marmoset cells transformed *in vitro* by EBV (17). The receptor appears to be qualitatively distinct from that induced by Herpes simplex, since it is capable of binding and forming rosettes with both human and rabbit immunoglobin coated erythrocytes whereas the HSV induced receptor forms rosettes with erythrocytes coated with rabbit but not human immunoglobin (Table III). The lines may be useful in detecting antigen-antibody complexes, or possibly in the purification and characterization of the chemical structure of this ligand receptor. We do not believe the IgFc receptor is induced by EB virus, since receptor activity is expressed on WM lymphocytes before transformation.

TABLE III. EXPRESSION OF THE IgFc RECEPTOR IN WOOLLY MONKEY LYMPHOBLASTOID LINES AND IN BURKITT LYMPHOMA LINES WITH AND WITHOUT HERPES SIMPLEX VIRUS INFECTION.

Cell Line	EBV Genome	HSV-1 Added	Fraction of Cells[b] making EA rosette: Rabbit 7S anti-SRBC	Fraction of Cells[b] making EA rosette: Human anti-D
WM	+	No	.88	.65
		Yes[a]	.90	.66
Raji	+	No	0	0
		Yes	.04	0
BJAB	−	No	0	0
		Yes	.02	
BJAB/HR-1 Clone B_1	+	No	0	0
		Yes	.03	0

[a] Multiplicity of 0.75 PFU/cell. Rosette formatio tested 24 hr after infection.

[b] In each rosette test, 100–200 cells were counted.

Several factors may correlate with the lac of oncogenicity of virus and transformed cell in this species of monkey. Firstly, WM lym phocytes are relatively refractory to transfor mation (Table 1). For example preparation of EBV which contain 10^4 transforming unit when assayed on human cells, will only trans form a fraction of the cultures when adde undiluted to WM cells. Since WM T lympho cytes spontaneously proliferate for severa weeks *in vitro,* we entertained the idea that T cells might suppress transformation. Howeve elimination of T lymphocytes and enrichmen for cells bearing the Fc receptor did not in crease the transformation rate (unpublished) The relative insusceptibility of lagothrix leu kocytes to transformation cannot be ex plained by the absence of virus receptor since we were, in fact, able to induce trans formation *in vitro,* albeit rarely. Ample num bers of lymphocytes with complement recep tors, thought to be closely associated with th virus receptor, are found in WM blood (Tabl II) (17), but the subpopulation of EAC ro sette-forming cells with virus receptors ma be small or the virus receptors may b sparsely distributed on WM B lymphocytes Susceptibility of cells to transformation an susceptibility of the host to infection may b related; higher transformation efficiencies o EBV are found in human cells, the natura host (18). Both the serologic studies and gen

eral failure to recover cell lines from peripheral blood indicate that WM are not infected following inoculation of virus or cells.

Secondly, WM lymphoblastoid lines are relatively nonproductive of virus. They are similar to lines derived from adult humans and they contrast sharply with transformed marmoset cells which release relatively large amounts of cell free virus (19). While autochthonous EBV transformed marmoset cells have proved oncogenic in one instance (20) the tumor may have been induced by virus released from the transformed marmoset cell and not by the transformed cells *per se.* In fact, though there is ample evidence for oncogenicity of the virus in marmosets, there is as yet no evidence that lymphoblastoid cells of any species, derived following in vitro exposure to EBV, are inherently tumorigenic.

Thirdly, transformed cells were apparently readily eliminated, despite the absence of preexisting anti-viral immunity. In one instance transformed cells were retrieved from the peripheral blood seven days after inoculation, but not thereafter, this suggests an efficient mechanism for recognition and elimination of transformed cells.

Summary. Lymphoblastoid cell lines were derived from peripheral blood leukocytes of woolly monkeys by exposure to the B95-8 strain of EB virus. Primary cells were relatively resistant to transformation and the resulting lines produced little virus. Almost half the lymphocytes in fresh blood had a receptor for IgFc and 60–90% of continuously cultured cells expressed receptors for both rabbit and human IgFc as detected with the "EA" rosette test. The IgFc receptor on woolly monkey cell lines was qualitatively different from that induced by herpes simplex virus which could be detected with rabbit but not human Ig. Monkeys inoculated with cell-free virus and autologous transformed cells remained well and showed no evidence of infection. There was thus no evidence that woolly monkey cells transformed *in vitro* by EBV were oncogenic when replaced in the autologous host.

We would like to thank Elizabeth Grogan for expert help with the HSV infection and rosetting experiments, Dr. James Robinson for characterization of the surface markers on primary and transformed cell lines, Dr. Roy Breg and Mrs. Vicki Fritz for help with preparation and interpretation of the karyotypes, Mrs. Muriel Lipman for electron microscopic studies, Mr. Walter Webb for his expert animal care and Mrs. Katherine Papov for excellent technical assistance.

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Serum LH Levels Following Multiple LHRH Injections in Aging Rats[1, 2] (40084)

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Reduced reproductive function is characteristic of aging in mammalian species. Although the precise neuroendocrine mechanisms involved with age-related alterations in reproduction remain unresolved, current information suggests that age may affect several components of the hypothalamic–hypophyseal–gonadal control system.

Recent studies have shown reduced serum testosterone levels in aged male rats and humans (1, 2). Ovarian steroid production also changes in aged rats. Normal ovarian cyclicity is replaced by periods of constant estrous and repetitive pseudopregnancies (3). The effects of age on gonadal function are related to the effects of aging on gonadotropin secretion. Although the reduction in gonadal steroid production with age in humans is accompanied by increased blood gonadotropin concentrations (4, 5), there is no evidence for similar increases in gonadotropin release accompanying the reduced gonadal steroid secretion in aged rats. We recently reported decreased serum LH levels in aged male rats (6). In female rats the effect of age on serum LH levels is variable and related to the variable ovarian states of the aged rat. Although aged constant estrous rats have higher serum LH concentrations than do young cycling female rats in estrous or diestrous stages of their ovarian cycles, the LH level in the aged rats is noncyclic and is much less than that found in young rats at proestrus (7). This study also showed that aged rats with repetitive pseudopregnancies or aged anestrous rats have serum LH levels which are lower than LH levels in young rats at diestrus. Additional studies have shown that ovaries from young rats grafted into aged female rats resume the endocrine state of the recipient before the transplant (3).

These studies suggest significant age effects on pituitary gonadotropin secretion. In most reproductive states old rats have decreased serum LH levels. These low LH concentrations suggest chronically reduced pituitary LH secretion. In addition we have reported smaller increases in serum LH following intravenous LH releasing hormone (LHRH) injections in aged compared to young rats of both sexes (6, 7). The increase in serum LH following acute LHRH injection in aged rats indicates that their pituitaries are capable of secreting greater amounts of LH than they normally maintain. The long-term reduced LH secretory activity which is characteristic of the aged rat could at least partially account for our reported decrease in pituitary responsiveness to actue LHRH in these animals. The current study was designed to consider the effects of more sustained LHRH stimulation on serum LH concentration in aging laboratory rats.

Materials and methods. The rats used in these studies were on the Long-Evans strain. Young adult animals were 4 and 5 mo of age, the aged groups ranged from 24 to 28 mo of age. The aged females were multiparous rats obtained as retired breeders at 9 mo of age (Blue Spruce Farms, Altamont, N.Y.). The young females and both groups of males were from the same lineage and had been reared in our colony. The rats were housed in a temperature controlled (22° ± 1°) and artificially lighted room (12 hr light cycle) and maintained on Wayne Lab-Blox and water supplied *ad libitum*. Female rat experimental groups included aged females with either constant estrous or persistent diestrous (repetitive pseudopregnant) vaginal smears and young females at estrus or diestrus day 2 of their ovarian cycles. Old rats were considered to be constant estrus or persistent diestrus if they had at least 10 consec-

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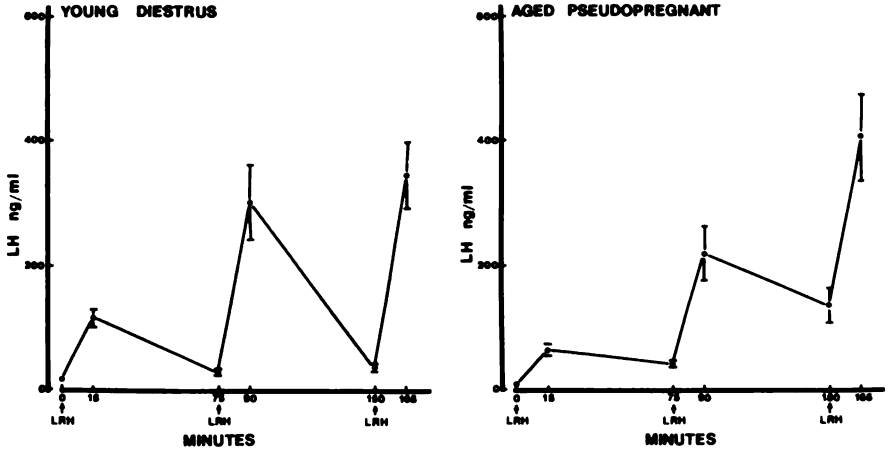
days of cornified or leucocytic vaginal
rs, respectively. All rats were under
h supervision of the Michigan State Uni-
ty Laboratory Animal Care veterinari-
The rats were maintaining stable body
ats and were free of any obvious acute
ronic disease.

periments were begun at 10:00 AM to
nize variation in pituitary response
h could be caused by diurnal patterns of
crine secretion. A pretreatment blood
le was taken by orbital sinus puncture
r light ether anesthesia. All experimental
os (12 rats/group) received three serial
venous injections of LHRH. Initially,
g of LHRH (Eli Lily, Inc., Indianapolis,
was injected into the exposed jugular
Serial blood samples averaging about 1
ere taken from each rat 15, 75, 90, 150
165 min after the first LHRH injection.
ar intravenous LHRH treatments (500
/ere also administered at the 75 and 150
blood sampling intervals. Serum LH was
ured by double antibody radioimmu-
says (8). The antibody used in these as-
was the NIAMD anti rat LH. The ref-
e hormone was NIAMD rat LH-RP 1.
e statistical analysis of the data (Michi-
State University CDC 6500 computer)
ded analysis of variance of the pretreat-
ment LH concentrations and multivariant analysis of variance to determine interaction between age groups, treatments, and treatment responses.

Results. Figure 1 compares serum LH levels in young, day 2 diestrous and aged pseudopregnant (persistant diestrous) female rats. Serum LH concentration in the samples taken prior to LHRH administration (0, 75, and 150 min) were different in the aged compared to the young groups ($P < 0.05$). LH was higher in the young group than the aged group at 0 time (16.3 vs 8.6 ng/ml). However, in the 150 min sample, LH levels in the aged group far exceeded that of the young rats (141.0 vs 35.7 ng/ml). Serum LH concentration was increased after each LHRH injection in both age groups ($P < 0.0001$). The increase in serum LH 15 min following each injection was greater in both the young and aged groups with each successive LHRH injection ($P < 0.01$). Although the increase in serum LH following the first LHRH injection was greater ($P < 0.05$) in the young than the aged group (112 vs 64 ng/ml), the differences between pretreatment and the 15 min postinjection LH values in the young and aged groups following the second and third LHRH injection were not different.

The effects of multiple LHRH injections



. 1. The effect of serial intravenous LHRH injections on serum LH concentration in young diestrous and ersistent diestrous female rats. ($n = 12$/group). LH is expressed as ng/ml of serum with indicated standard of the means. Serial samples were taken under light ether anesthesia before LHRH injection (0, 75, and 150 and 15 min following LHRH stimulation (15, 90, and 165 min). Serum LH was higher in young than the group at the 15 min sample ($P < 0.05$). The increase in LH was greater after the second and third LHRH on in both groups ($P < 0.01$). The pretreatment LH concentrations were increased prior to the second and .HRH injections in the aged group ($P < 0.05$).

on serum LH concentrations in young estrous and aged constant estrous female rats are illustrated in Fig. 2. LH levels in the blood samples taken from the aged group before each LHRH injection (0, 75 and 150 min), increased with each sampling interval ($P <$ 0.01). Mean LH concentrations increased from 13.5 to 79.1 and 207.9 ng/ml from the 0 min to the 75 min to the 150 min sampling interval. Serum LH was increased after each LHRH injection in both age groups ($P <$ 0.0001). The increase in LH in the 15-min sample was greater for the young than the aged groups ($P < 0.01$). Although the increases in serum LH concentrations in the young group were similar after the second and third LHRH injection to the response after the first injection, the aged group had greater increases in LH after the second and third LHRH injection (the increase in serum LH after the first injection was 163 ng/ml; after the second, 474 ng/ml; and after the third, 479 ng/ml). In addition, the increases in LH were similar after the second and third LHRH injections in the young and aged groups.

The response of young and aged male rats to the three injections of LHRH is plotted in Fig. 3. Although the mean preinjectio levels of the young male group were co ently higher than LH in the aged males vs 27.0 ng/ml at 0 min; 40.1 vs 21.8 r at 75 min; and 32.6 vs 27.8 ng/ml a min), these differences were not signif Both age groups had increased serun after each LHRH injection ($P < 0.0001$ though the increase in LH after the LHRH injection was greater ($P < 0.05$) i young than in the aged group (182.0 vs ng/ml in the 15-min samples), the inc in LH over the preinjection hormone was similar after the second and third L injections for both age groups.

Discussion. These data suggest subst differences in young and aged rat pitu response to multiple LHRH stimula Levels of serum LH measured after the i LHRH injection were uniformly low aged compared to young experim groups. This finding is in agreement wit recent reports in aged rats of both sex 7). However, the results of this study that age-related differences in serum L much less prominent following mu LHRH stimulations. The marked incre serum LH following the second and

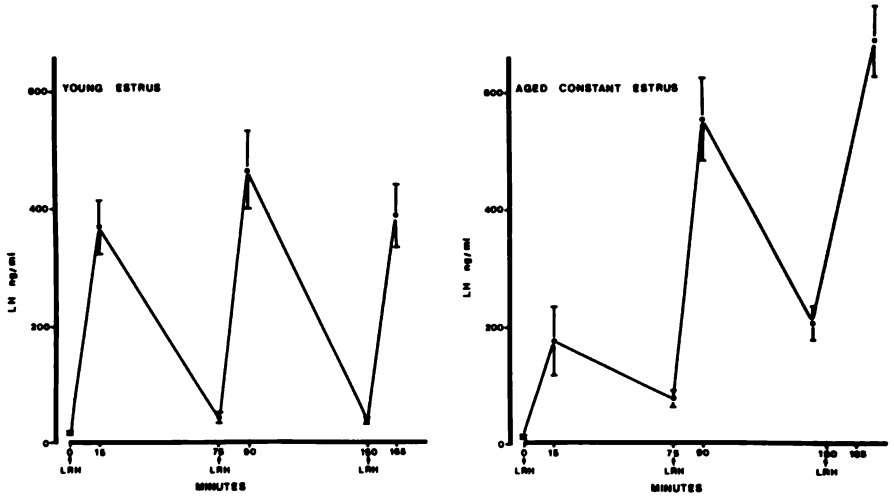
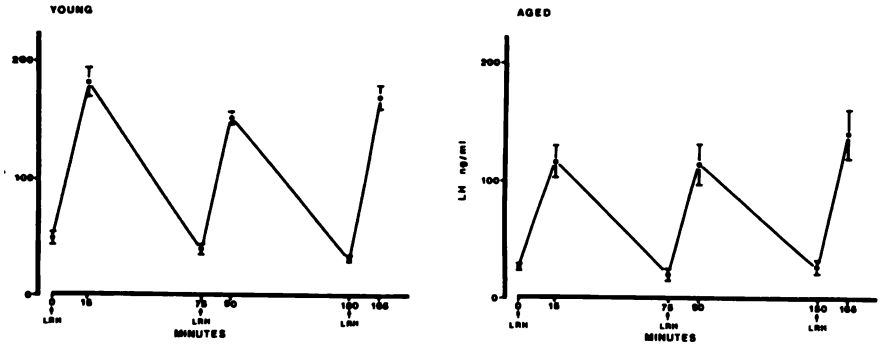


FIG. 2. The effect of serial intravenous LHRH injections on serum LH concentration in young estrous an constant estrous female rats (n = 12/group). LH is expressed as ng/ml of serum with indicated standard error means. Serial blood samples were taken under light ether anesthesia before LHRH injection (0, 75, and 15 and 15 min following LHRH stimulation (15, 90, and 165 min). Serum LH was higher in young than the aged at the 15 min sample ($P < 0.01$). The increase in LH was greater after the second and third LHRH injections aged group ($P < 0.01$). The pretreatment LH concentrations were increased prior to the second and third l injections in the aged group ($P < 0.01$).



The effect of serial intravenous LHRH injections on serum LH concentration in young and aged male 12/group). LH is expressed as ng/ml of serum with indicated standard error of the means. Serial blood ere taken under light ether anesthesia before LHRH injection (0, 75 and 150 min) and 15 min following mulation (15, 90, and 165 min). Serum LH was higher in the young than the aged group at the 15 min < 0.05).

treatments in the aged female rats young diestrus female group is similar cepted self-priming effects of LHRH ly shown to be involved in the proes- surge. It is presumed that the in- responsiveness to LHRH involves in gonadal steroids which affect pi- esponsiveness to LHRH (9), to the ning effects of LHRH on pituitary etion (10) which results in increased thesis or secretory processes within gonadotrophs. The marked increase LH levels in the preinjection blood of the aged female groups suggest more sustained LH release following or more likely, a slower rate of re- f the hormone from the circulation in imals.

ugh the current study indicates that l rat can sustain substantial serum ls under experimental conditions of RH stimulation for limited time pe- ere is ample evidence that the re- ness of the hypothalamic-hy- al unit to LH controlling input is l in the aged rat. We and others have low basal serum LH levels in aged ts (6, 9) and in aged female rats in reproductive states (7, 11). A recent uggests that serum LH levels during strous surge are reduced in 12 mo ats compared to 3–5 mo old cycling). In addition, it has been reported uitary concentrations of LH are in aged male and female rats com- young adult controls (13, 14). These low blood and pituitary gonadotropin concentrations have been implicated in the decrease in reproductive performance of aged rats of both sexes (1, 3, 15). We have shown decreased serum testosterone levels in aged male rats (1). If the reproductive control systems of these aged males were functionally normal, this decrease in testosterone would be detected in the hypothalamic–hypophyseal feedback system and result in increased LH secretion. Since aged male rats have increased serum testosterone after HCG injection, indicating that they are capable of responding to gonadotropin, we conclude that the reduced serum testosterone in aged male rats reflects reduced gonadotropin stimulation of the testis.

We and others have hypothesized that the most fundamental age-related alteration in the reproductive control system occurs in the neural regulation of hypothalamic hormone release (3, 5, 17). Hypothalami of aged male rats contain sufficient gonadotropin releasing activity to stimulate LH release from incubated rat pituitaries (13). Aged male and female rats have smaller increases in serum LH and FSH following orchidectomy and ovariectomy (9, 18). These findings indicate that although the reproductive control system in aged rats respond to decreased negative feedback following gonadectomy, the response is less than that which occurs in the young adult rat. The constant estrous state in aged female rats seems involved with failure of hypothalamic mechanisms responsible for the proestrous LH surge. The preoptic

area of the rat hypothalamus contains gonadotropin releasing hormone (19). Electrical stimulation of this region can cause ovulation in rats (20) and lesions of the preoptic area have been shown to reduce gonadotropin releasing hormone content in this region (21) and cause persistent vaginal cornification in young female rats (19). In addition, the preoptic area has been reported to be the site of the stimulatory effect of estrogen on gonadotropin release (22). Aged constant estrous rats have sufficient serum LH levels to stimulate minimal follicular development and estrogen secretion (23). Although electrical stimulation of the preoptic area under these conditions will stimulate ovulation (24), the hypothalamic mechanisms have apparently become less responsive to endogenous estrogen stimulation of gonadotropin release. The results of the present study show that aged rats can sustain high levels of serum LH if the hypothalamic regulation of LH release is circumvented by the injection of LHRH, suggesting that the ability of the hypothalamus to secrete gonadotropin releasing factors is impaired in the aged rat.

Although the precise mechanisms involved in the deterioration of hypothalamic function in the aged rat are not understood, a substantial amount of experimental data suggests that hypothalamic catecholamines may be involved. Hypothalamic catecholamines have been shown to influence anterior pituitary secretions presumably either by affecting the release of hypothalamic pituitary regulating hormones or by the catecholamines acting directly on the pituitary. Increased hypothalamic catecholamines have been strongly implicated in the release of pituitary LH and the inhibition of pituitary prolactin release (25). The decrease in serum LH and increase in serum prolactin we have found in the aged rat is consistent with a hypothesis of decreased hypothalamic catecholamine function. This hypothesis is supported by reports of decreased hypothalamic catecholamine content (26, 27) and reports of decreased neuronal catecholamine turnover rates in aged rats and mice (26, 28). However, a great deal more experimentation is required to understand the molecular basis for changes in hypothalamic catecholamine function in aging rats and to understand how changes in hypothalamic catecholamines may be related to alterations in hypothalamic responsi to multiple stimulatory and inhibitory it receives.

Summary. The effect of three serial tions of 500 ng of LHRH on serum L tested in young (4–5 mo) and aged (mo) male and female Long–Evans rats female groups were rats showing cc estrous or pseudopregnant vaginal cy (at least 10 days of cornified or leu vaginal smears, respectively) which compared to young female rats at estro diestrous stages of their ovarian cycl increase in serum LH following th LHRH stimulation was less in the age in the young groups. Serum LH con tions in the aged female group were pr sively increased following the secon third LHRH injections. The increase rum LH following the second and LHRH injections was similar in your aged female groups. LH concentrati blood samples collected before LHRH tion was progressively increased in bot female groups. Although LH was gre young than aged males after the first I injection, LH concentrations after the s and third injections were similar in bot These results indicate that aged rat pitu can sustain higher serum LH concentr than are normally found in their circu and suggest that failure of hypothalami monal stimulation of LH release contr to the loss of reproductive function in The increase in LH following m LHRH injections in aged female rats r increased pituitary responsiveness foll the priming effect of the first stimulatic reduced LH clearance.

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Squamous Metaplasia of the Tracheal Epithelium in Organ Culture. II. Nutritional Influences[1] (40085)

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Squamous metaplasia is defined as the replacement of differentiated epithelial cells by squamous-like cells not normally present at that tissue site (1). In the respiratory mucosa, metaplasia is a common response of the epithelium to a variety of mechanical (2, 3), chemical (4, 5) and metabolic stimuli (6).

Although usually considered a benign and reversible lesion, squamous metaplasia occasionally is associated with squamous cell carcinoma *in vivo* and may, under some circumstances, constitute a distinct pre-cancerous stage in the development of this lesion. Consequently, factors affecting epithelial differentiation, and in particular, squamous metaplasia, may contribute to neoplastic transformation in some as yet unknown fashion.

Vitamin A is essential for the maintenance of a well differentiated respiratory mucosa (6, 7). In its absence, the mucociliary epithelium becomes metaplastic and keratinized. Experiments in our laboratory indicate that other nutritional influences may also play a role in the development of squamous metaplasia (8, 9). When cultured in a vitamin A-deficient, nutritionally complex medium such as Waymouth's MAB 87/3, the differentiated respiratory mucosa of the hamster trachea rapidly undergoes squamous metaplasia and keratinization. In contrast, these explants exhibit a differentiated columnar epithelium for extended periods of time when maintained in a basal medium such as Eagle's minimum essential medium (MEM) in the absence of vitamin A.

Studies reported here were undertaken to determine the nutritional constituents in Waymouth's medium, but absent from Eagle's MEM, that promote squamous metaplasia.

Materials and methods. Organ Culture Preparation. Hamster tracheal explants were prepared from 6-week-old inbred female hamsters (87.20 strain, TELACO, Bar Harbor, ME) by previously described methods (8, 9). The trachea was excised from the larynx to the bronchial bifurcation and immediately immersed in Hanks' balanced salt solution containing 100 μg Gentamicin and 50 u nystatin per ml. The trachea was then opened along the anatomic discontinuity in the cartilage rings, and individual rings were cleaved from the tissue. Each resulting C-shaped segment was cut in half to yield explants with an epithelial surface approximately 1 × 2 mm. A segment of tissue from each animal was fixed in Bouin's solution before culturing. The remaining pieces were then placed into a 35 mm plastic Petri dish and 0.4 ml of a test medium containing 50 μg Gentamicin and 25 u nystatin per ml. Explants were maintained in a 95% air–5% CO_2 water-saturated environment at 35° and the medium changed twice weekly.

Formulation of media. Table I lists the nutritional constituents present in Waymouth's MAB 87/3 medium (10) but absent from Eagle's MEM (11). These components were divided into 5 groups on the basis of their chemical or nutritional similarities. Group I consisted of insulin and glutathione. Group II contained the three water soluble vitamins, ascorbic acid, B_{12}, and biotin. Group III was made up of the nucleic acid intermediates thymidine and hypoxanthine. Group IV was composed of eight inorganic salts (KH_2PO_4, $MgCl_2 \cdot 6H_2O$, $FeSO_4$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7\ H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ and $CoCl_2 \cdot 6H_2O$). Group V consisted of eight nonessential amino acids (L-alanine, L-asparagine, L-aspartic acid, L-cysteine HCl, L-glutamic acid, glycine, L-proline, L-serine). Test media were formulated by adding each nutritional constituent, or group of constituents, to Eagle's MEM at the same concentrations as found in Waymouth's MAB 87/3. Since there are substantial differences between the two media in

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TABLE I. CONSTITUENTS PRESENT IN WAYMOUTH'S MAB 87/3 BUT ABSENT FROM EAGLE'S MINIMUM ESSENTIAL MEDIUM (MEM).[a]

		Mg/L
Group I	INSULIN AND GLUTATHIONE	8.00
	Insulin	15.00
	Glutathione	
Group II	VITAMINS	
	Ascorbic acid	17.50
	Vitamin B_{12}	.20
	Biotin	.02
Group III	NUCLEIC ACID INTERMEDIATES	
	Thymidine	8.00[b]
	Hypoxanthine	25.00
Group IV	INORGANIC SALTS	
	KH_2PO_4	208.0
	$MgCl_2 \cdot 6H_2O$	240.0
	$FeSO_4$	.45
	$CuSO_4 \cdot 5H_2O$	.50
	$MnSO_4 \cdot H_2O$	.016
	$ZnSO_4 \cdot 7H_2O$	.030
	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	.025
	$CoCl_2 \cdot 6H_2O$	.022
Group V	NONESSENTIAL AMINO ACIDS	
	L-Glutamic acid	150.0
	L-Aspartic acid	60.0
	L-Serine	12.80[b]
	Glycine	50.00
	L-Proline	50.00
	L-Cysteine-HCl	90.00
	L-Alanine	11.20[b]
	L-Asparagine	24.00

[a] Formulations in GIBCO catalog, (1973–1974), Grand Island, NY, pp. 110, 120.
[b] Corrections to original formulation.

the concentrations of glucose, riboflavin, thymine and choline chloride, the concentrations of these four constituents were adjusted to the higher concentration in the Waymouth's formulation. Media were sterilized by Millipore filtration under nitrogen pressure and osmolality was determined with a Fiske Osmometer. The experimental media ranged between 285 and 310 mOsm/kg water. Since these values were within the normal range of osmolalities found in commonly used tissue culture media (12), adjustment of experimental media was not considered necessary.

Histological techniques. Explants from each experimental group were harvested after 4 weeks, fixed in Bouin's solution, dehydrated in alcohol and embedded in paraffin. Sections (5 μm) were stained using hematoxylin and eosin and evaluated according to the following morphologic criteria. Specimens exhibiting a ciliated columnar epithelium that showed no metaplastic changes were classified as differentiated respiratory epithelium (Fig. 1A). If metaplastic changes affected less than 15% of the total epithelial surface, it was described as focal squamous metaplasia (Fig. 1B). Specimens with greater than 15%, but less than 50% metaplasia were designated as moderately metaplastic, whereas those lesions affecting greater than 50% of the epithelium were defined as extensive (Fig. 1C). Explants were assigned a numerical score from one (differentiated) to four (extensive metaplasia) to make quantitative characterization of groups feasible. Keratinization, identified as accumulations of flattened, desquamated, nucleated or anucleated surface cells, was also noted (Fig. 1D).

Results. Addition of Single Groups of Nutrients. In a series of parallel experiments, explants were cultured in: (a) MEM, (b) MEM with one of the individual groups of constituents added (Groups I–V), (c) MEM plus all five groups of additives (simulated Waymouth's MAB 87/3) or (d) the nutritionally complex medium, Waymouth's MAB 87/3. Nineteen to 38 specimens from each experimental group were graded; the inclusion of an equal number of explants for each test group in the analysis was not possible because of sporadic fungal contamination of cultures and imperfections in histologic preparations.

Figure 2A indicates the relative proportion of the specimens with metaplastic changes after 4 weeks. Explants cultured in MEM usually showed a differentiated mucociliary epithelium, whereas those maintained in Waymouth's MAB 87/3 or the medium fabricated to simulate Waymouth's (MEM plus Groups I–V) exhibited extensive metaplasia. The addition of insulin and glutathione (Group I) or the vitamins (Group II) to MEM failed to enhance the development of squamous metaplasia. On the other hand, Group III (the nucleic acid intermediates), Group IV (the inorganic salts), and Group V (the nonessential amino acids) produced an apparent increase in metaplasia.

Due to the ordinal nature of the histologic assessment, a nonparametric multiple comparison procedure was used to search for differences in squamous metaplasia between pairs of experimental groups at the 95% simultaneous level of confidence (13). A significant increase in metaplastic activity was found with use of Waymouth's medium, the

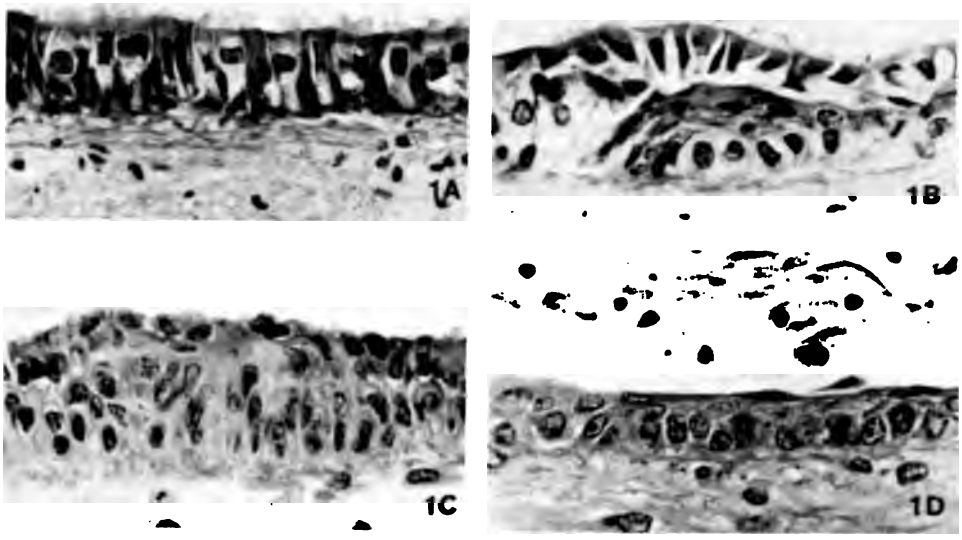

FIG. 1. Examples of morphologic criteria for evaluation of explants. A) Differentiated columnar epithelium, ×1160. B) Focal basal cell metaplasia, MEM with addition of proline, ×1112. C) Diffuse squamous metaplasia, MEM with addition of Groups I–V, ×1080. D) Squamous metaplasia with keratinization, MEM with addition of Groups I–V, ×1120. Explants were maintained in culture for 4 weeks.

simulated Waymouth's medium and MEM plus Group V (as compared to MEM).

Similar effects were observed when keratinization was evaluated. Thus, when Group V was added to MEM, the results did not differ significantly from those obtained with Waymouth's and the simulated Waymouth's medium. Keratinization in explants maintained in MEM with either Group III or Group V was more prominent than with MEM alone. These differences were statistically significant (14, 15).

Addition of groups of nutrients in combination. Because Groups III, IV, and V ranked nearest to Waymouth's in the induction of metaplasia and keratinization, combinations of the three were investigated to determine whether or not their effects were additive (Fig. 2B). Differences between combinations were examined using metaplasia and keratinization as a basis for assessment (13, 15). Although the nucleic acid intermediates of Group III and the inorganic salts of Group IV appeared to produce an increase (not statistically significant) in metaplasia when added individually to MEM (Fig. 2A), metaplasia was not more prominent when the two groups were combined (Fig. 2B). Similar results were obtained when both Group IV and Group V were added to MEM. Increased degrees of metaplasia and keratinization developed in explants maintained in MEM with Groups III and V and in those cultures in the simulated Waymouth's medium. We conclude from these observations that Group IV (the inorganic salts) does not contain essential nutritional constituents required for the development of squamous metaplasia and keratinization.

Addition of individual amino acids. Because the nonessential amino acids comprising Group V were most effective in promoting squamous metaplasia and keratinization, an attempt was made to determine whether an individual amino acid or several different amino acids induced these changes. Each of the amino acids (L-alanine, L-asparagine, L-aspartic acid, L-cysteine HCl, L-glutamic acid, glycine, L-proline and L-serine) was added individually to MEM at a concentration of 3.72 mM (i.e, the total concentration of nonessential amino acids in complete Waymouth's medium).

The combined results of duplicate experiments are presented in Table II. Extensive squamous metaplasia and keratinization de-

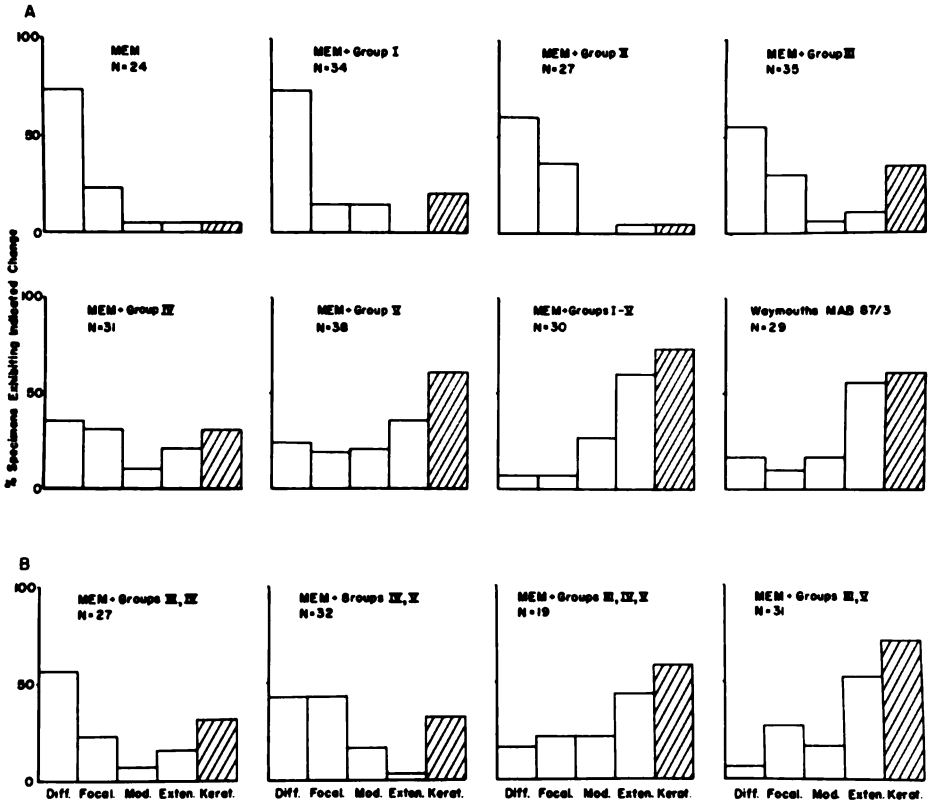

FIG. 2. Features of the epithelium in hamster tracheal organ cultures 4 weeks after maintenance in Eagle's EM with supplementation. A) Single group additive experiments. B) Combination group experiments. The rcentage of the epithelium showing metaplasia was graded on observance of five or more serial sections from ch tissue. N = numbers of explants graded in each group.

TABLE II. ADDITION OF NON-ESSENTIAL AMINO ACIDS TO MEM. PERCENTAGE OF SPECIMENS EXHIBITING INDICATED CHANGES.[a]

	Experiment I					
Amino acid (3.72 mM)	No. Exam.	Diff.	Focal	Mod.	Exten.	Kerat.
L-Alanine	26	61	39			7
L-Asparagine	25	80	20			0
L-Aspartic acid	35	74	26			2
L-Cysteine HCl	25	100				0
L-Glutamic acid	40	55	23	13	10	18
Glycine	29	44	51	3		3
L-Proline	31	74	26			3
L-Serine	23	61	17	18	4	22
L-Serine (1.86 m*M*)	22	73	9	5	14	18
L-Serine (7.44 m*M*)	14	86	14			7
L-Serine (18.5 m*M*)	18	28	67	6		6
Group V	56	27	19	18	32	48
Mem	24	71	21	4	4	4

[a] After 4 weeks in culture, the percentage of the epithelium exhibiting squamous metaplasia and keratinization as graded independently by two observers as described in the text.

eloped only in explants maintained in me-ium supplemented with either L-glutamic id or L-serine. Epithelia cultured in MEM ith the addition of L-alanine, L-asparagine, L-aspartic acid, L-cysteine or L-proline failed to develop metaplasia affecting greater than 15% of the epithelium. The majority of these cultures exhibited a differentiated mucosa.

Obvious necrosis of epithelial cells and collapse of the cartilage ring were seen in explants cultured in L-cysteine. Cell death has been noted by others (16) in monolayer cultures exposed to cysteine at concentrations of > 1.0 m*M*.

L-Serine was added to MEM at different concentrations (1.86, 3.72, 7.44 and 18.5 m*M*) to determine whether or not its effects were dosage-dependent. At all concentrations but the lowest, cytotoxic changes were evident in explants.

Discussion. In the absence of either serum or vitamin A, the differentiated respiratory mucosa undergoes squamous metaplasia when cultured in an enriched synthetic medium such as Waymouth's MAB 87/3. Since these changes do not develop in a basal medium such as Eagle's MEM or Dulbecco's medium (8), one is obliged to conclude that the nutritional additives in the more complex medium stimulate cellular proliferation and squamous metaplasia. This investigation was undertaken to define the specific nutritional supplements responsible for these effects.

Although studies using serum-free chemically defined media have contributed to our understanding of the nutritional requirements of continuous lines of mammalian cells, a paucity of information exists concerning the growth requirements of primary cell or organ cultures (17). Since the epithelium of tracheal organ cultures is comprised of a diverse population of cells, the specific nutritional needs of each cell type are difficult to assess. Moreover, little is known concerning the relationship of cellular nutrition to differentiation.

It would appear that nutrients possessing the capacity to stimulate cell growth are important in the generation of squamous metaplasia in respiratory mucosa. Since the vitamin A stores in the epithelium probably are limited, the manifestation of vitamin A deficiency develops readily. Our observations indicate that nucleic acid intermediates, in combination with nonessential amino acids, contribute significantly to the development of squamous metaplasia in vitamin A-deprived tracheal cultures. This might be related to a nonspecific growth-stimulatory effect of these constituents as is observed when they are added to monolayer cultures (18, 19). *Possibly the de novo* synthesis of nucleic acid intermediates requires expenditures of energy by the cell that can be alleviated in part by the addition of preformed intermediates.

The most pronounced enhancement of squamous metaplasia and keratinization was produced by the addition of nonessential amino acids to the culture medium. Although termed "nonessential" *in vitro* as they are not required for the growth of strain L or HeLa cells in monolayer culture (11), these amino acids influence protein synthesis and energy metabolism (17). They can be incorporated directly into proteins, utilized as biosynthetic precursors for important regulatory metabolic intermediates, or serve as allosteric effectors of various anabolic reactions stimulating cell proliferation (20). Although several laboratories report that specific essential amino acids stimulate protein synthesis and mitotic activity in cell cultures (21–23), little work has been done to evaluate the effects of individual nonessential amino acids on growth and differentiation. In monolayers of fetal muscle cells, Matheson and Pahira (24) documented an increase in myotube formation and an approximate doubling of DNA and protein synthesis with addition of all nonessential amino acids to MEM. Similarly, Griffiths and Pirt (25) have observed that a mixture of serine, alanine and glycine increases the cell yield in suspension cultures of mouse LS cells when added to medium containing essential amino acids.

In our experiments, L-serine and L-glutamic acid had more striking effects on metaplasia and keratinization than other nonessential amino acids. These observations can be related to nutritional studies using cells in monolayer. Phytohemagglutinin (PHA) or antigen-stimulated human lymphocytes (26), rabbit fibroblasts (27) and cells from the limb-buds of rabbit embryos (11) have a requirement for serine in addition to essential amino acids. In clonal populations of HeLa cells, nonessential amino acids also become essential for optimum growth and survival (28). In these experiments, the addition of serine alone is able to replace the group mixture. Presumably, under normal circumstances, serine is lost from the intracellular pool into the medium at a rate exceeding the capacity of the cells to synthesize the amino acid (11).

utamine is the growth-limiting essential
o acid in most cell cultures because of
pid uptake by the cells and its instability
ılture ınedium (29). However, cells can
dapted to growth at high levels of glu-
ɔ acid when substituted for glutamine
Demars has observed increased amounts
e enzyme glutamine synthetase in these
ɹes (31).
sulin and glutathione did not promote
mous metaplasia in explanted tracheal
ɛ. Although insulin stimulates growth in
ɨ mammalian cell lines (32), insulin and
thione failed to affect epithelial differ-
tion in our experiments. The vitamins
seemed unimportant. Since the amounts
ıese components required for maximal
th of many cell lines are small (32),
lementation probably does not affect
th in an additive fashion. Ascorbic acid,
ıin B_{12} and biotin are not absolute re-
ɛments for the sustained growth of mam-
an cells *in vitro* (30).

ɯmmary. The mucociliary epithelium of
ıdult hamster trachea undergoes squa-
s metaplasia in organ culture when main-
d in a complex, chemically-defined me-
ı (Waymouth's MAB 87/3) without se-
This change fails to develop in explants
ıred in Eagle's minimum essential me-
ı (MEM) (8, 9). To determine the factors
ıoting squamous metaplasia, nutritional
ɨituents in Waymouth's MAB 87/3 me-
ı but not present in MEM were divided
five groups: (a) insulin and glutathione,
ɾitamins C, B_{12} and biotin, (c) nucleic
intermediates, d) inorganic salts and e)
ssential amino acids. Test media were
ulated by adding each group or combi-
ɔns of groups to Eagle's MEM. Organ
ıres were maintained in the test media
weeks and then examined histologically.
nonessential amino acids were most im-
ınt in enhancing metaplasia and keratin-
ɔn. When nonessential amino acids were
d individually to MEM, L-glutamic acid
L-serine seemed most influential in in-
ıg metaplastic changes.

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ements and Herman C. West.

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Intracellular Calcium: Lack of Effect on Ovine Red Cells (40086)

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Although human red cells normally have only small amounts of intracellular Ca^{2+} (ca. 20 μM), cellular Ca^{2+} concentrations may be greatly increased through incubation of human erythrocytes with the ionophore, A23187 ("Calcimycin"). A23187 allows the free passage of divalent cations, notably Ca^{2+} and Mg^{2+}, across biological membranes until equilibrium is established (1–3). Such artificial Ca^{2+} loading of human and animal erythrocytes does extensive damage as reflected by (i) marked shape changes; (ii) concomitant loss of cell water and K^+; (iii) increased mean cell hemoglobin concentration (MCHC); (iv) profound and rapid hydrolysis of intracellular adenosine triphosphate (ATP), presumably due to stimulation of membrane Ca^{2+} efflux ATPase; (v) decreased osmotic fragility; and (vi) stiffening of the red cell membrane (2–10). We have suggested that similar Ca^{2+}-induced cellular damage may underlie the formation of irreversibly sickled cells in patients with sickle cell disease (6, 7, 9, 11). These permanently deformed cells have abnormally large amounts of Ca^{2+} and possess most of the characteristics of Ca^{2+}-laden normal erythrocytes, being dehydrated, distorted and energy depleted (11–13).

We have since determined that red cells from diverse species of mammals, including rats, mice, rabbits and pigs, respond as do human erythrocytes to ionophore-induced Ca^{2+} influx. Most recently, we have investigated the effects of elevated intracellular Ca^{2+} on red cells from newborn and adult sheep. Whereas most red cells from newborn sheep are Ca^{2+} responsive, those from adult sheep are not.

Materials and methods. In all experiments reported here, heparinized blood from sheep and humans was kept on ice and used within 4 hr of venipuncture. Intracellular Ca^{2+} concentrations were measured on saline washed erythrocytes by atomic absorption spectroscopy as described earlier (11). ATP was measured by the technique of Beutler (14).

Incubations with ionophore. In all experiments, erythrocytes were washed 3 times with, and resuspended in, Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, 20 mM in Tris (tris[hydroxymethyl]aminomethane). At the beginning of each incubation, 50 μM A23187 was introduced, dissolved and diluted as previously reported (9). Incubations were conducted for 60 min at 37° with cell suspensions of hematocrit 35% (v/v). Ca^{2+}, when present, was added to a final concentration of 50 μM. Control samples had no added Ca^{2+} and contained 1 mM ethyleneglycol-bis-(α-amino-ethyl ether)N,N'-tetraacetic acid (EGTA) to chelate any residual Ca^{2+}.

Ca^{2+} ATPase assays. Ca^{2+} ATPase activity was estimated in crude lysates of thrice saline washed, packed erythrocytes, lysed by sonic disruption and by freezing and thawing once. Control experiments indicated that, although Ca^{2+} ATPase activity requires the presence of membranes, washing red cell membranes after lysis causes progressive diminuation of Ca^{2+} ATPase activity. Reaction mixtures contained 0.28 ml of crude lysate, 0.2 ml of 100 mM Tris buffer, pH 7.2, containing 2 mM $MgCl_2$, 0.02 ml of 30 mM ouabain (to inhibit Na^+/K^+ ATPase), 0.02 ml of 60 mM ATP, 0.01 ml of 500 μM A23187 (to ensure availability of Ca^{2+} to membrane ATPase sites), and either 0.02 ml of 100 mM neutralized EGTA or 0.02 ml of 1.5 mM $CaCl_2$ (total volume of reaction mixture = 0.60 ml). ATPase activity was determined by measuring the ATP content of the reaction mixtures at 0 time and after 60 min incubation at 37° (during which time the rate of ATP hydrolysis was approximately linear). Conditions of assay, including concentrations of Mg^{2+}, Ca^{2+}, EGTA and ATP were experimentally optimized for human erythrocyte Ca^{2+} ATPase.

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Results. The effects of ionophore-induced Ca^{2+} influx upon human and adult sheep erythrocytes are strikingly discrepant. Human erythrocytes, during 60 min incubation with A23187 and Ca^{2+}, become "spheroechinocytic" in shape, lose volume, become osmotically less fragile and hydrolyze most of their intracellular ATP (Fig. 1A and 1B, Table I).

In contrast, erythrocytes from adult sheep do not change shape (Fig. 1C and 1D), show no detectable increase in MCHC, retain their original osmotic fragility and hydrolyze relatively little of their original ATP (Table I). The lack of response of sheep red cells to Ca^{2+} influx is not due to an insensitivity of these cells to the Ca^{2+} ionophore; sheep erythrocytes, which normally have low intracellular Ca^{2+}, accumulate almost as much Ca^{2+} as do human red cells during 60 min incubation with A23187 and Ca^{2+} (Table I). In other experiments, the inclusion of as much as 10^{-3} *M* Ca^{2+} elicited no response from red cells of adult sheep in any of these parameters. Furthermore, sheep red cells previously ATP depleted by incubation with sodium fluoride do not change shape or shrink when subsequently incubated with A23187 and Ca^{2+}. Similarly ATP depleted human red cells retain their tendency to shrink and

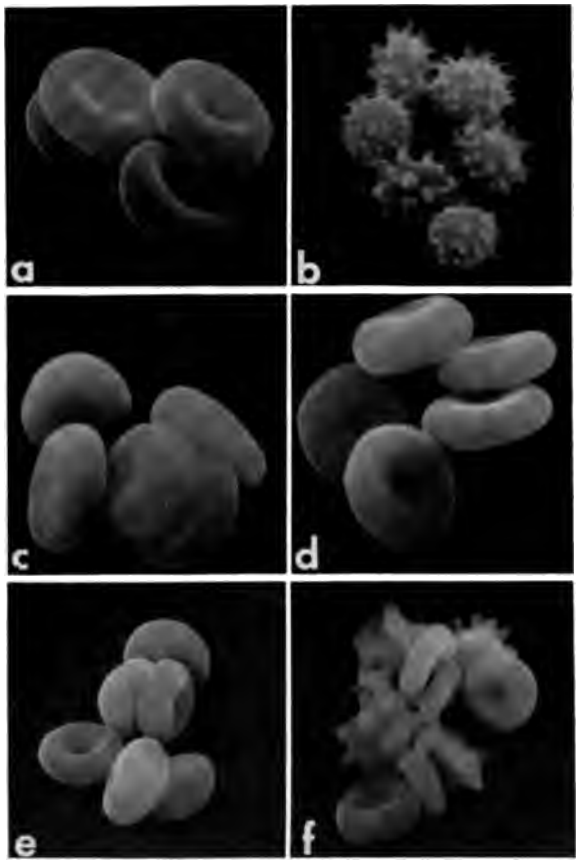

FIG. 1. Scanning electron micrographs showing morphologic effects of ionophore-induced Ca^{2+} accumulation on human, adult sheep and newborn lamb red cells. Human erythrocytes, incubated with A23187 and EGTA, remain normally shaped biconcave discs (A) (× 2900), whereas incubation with A23187 and Ca^{2+} causes the formation of spiky "spheroechinocytes" (B) (× 2500). Similarly treated adult sheep red cells remain biconcave discs whether exposed to A23187 and EGTA (C) (× 4500) or Ca^{2+} (D) (× 4200). Erythrocytes from newborn lambs, although morphologically normal in the presence of A23187 and EGTA (E) (× 3000), become spheroechinocytes when exposed to A23187 and Ca^{2+} (F) (× 2500). All cells were fixed in phosphate buffered formalin after 60 min incubation. Other experimental conditions as given in the legend to Table I.

TABLE 1. CHARACTERISTICS OF HUMAN, ADULT SHEEP AND NEWBORN (24-hr OLD) LAMB RED CELLS BEFORE AND AFTER INCUBATION WITH A23187 AND EGTA OR ADDED Ca^{2+}. VALUES IN PARENTHESES REPRESENT ±1 SD.

Characteristic	Human (n = 12)	Adult sheep (n = 12)	Newborn sheep (n = 5)
MCHC (gm Hb/dl RBC)			
A. Unincubated[a]	35.1 (±1.0)	34.7 (±1.2)	33.7 (±1.9)
B. A23187 + EGTA[b]	34.4 (±1.2)	34.7 (±1.3)	33.5 (±0.7)
C. A23187 + Ca^{++}[c]	44.6 (±3.1)	34.5 (±1.1)	41.2 (±3.0)
ATP (μM/gm Hb)			
A. Unincubated	3.85 (±0.51)	2.43 (±0.42)	2.15 (±0.30)
B. A23187 + EGTA	3.70 (±0.60)	2.15 (±0.64)	1.78 (±0.58)
C. A23187 + Ca^{++}	0.60 (±0.53)	1.84 (±0.53)	0.51 (±0.32)
Osmotic fragility (%NaCl @ 50% hemolysis)			
A. Unincubated	0.43 (±0.02)	0.62 (±0.03)	—
B. A23187 + EGTA	0.43 (±0.04)	0.61 (±0.04)	—
C. A23187 + Ca^{++}	0.28 (±0.04)	0.64 (±0.05)	—
Intracellular Ca^{++} (μM/l RBC)			
A. Unincubated	24.3 (±9.1)	29.5 (±6.5)	31.1 (±8.4)
B. A23187 + EGTA	<10	<10	<10
C. A23187 + Ca^{++}	160.2 (±27.1)	138.3 (±21.1)	—
Predominant cell shape			
A. Unincubated	biconcave discs	biconcave discs	biconcave discs
B. A23187 + EGTA	biconcave discs	biconcave discs	biconcave discs
C. A23187 + Ca^{++}	spheroechinocytes	biconcave discs	spheroechinocytes (≈70%)

[a] Fresh red cells.
[b] Red cells incubated 60 min with A23187 and EGTA.
[c] Red cells incubated 60 min with A23187 and Ca^{2+}.

change shape when Ca^{2+} loaded (9). Fluoride-induced energy depletion alone has no effect upon the shape of either human or sheep erythrocytes. Therefore, the insensitivity of sheep red cells to intracellular Ca^{2+} is not explained by a failure of these cells to accumulated Ca^{2+}, or by insufficient Ca^{2+} concentrations in the incubation medium, or simply by their failure to become ATP depleted.

Suspension of human erythrocytes in isotonic KCl during ionophore-induced Ca^{2+} loading will block both cellular shrinkage and shape change (but not ATP hydrolysis) (9). This is presumably due to inhibition of passive cellular K^+ loss by the high extracellular K^+ concentrations. Sheep are polymorphic with regard to red cell K^+ content, and some have high Na^+ and low K^+ erythrocytes (15, 16), apparently due to a deficiency of membrane $Na^+ - K^+$ ATPase (17, 18). Using red cells from 3 high K^+ and 3 low K^+ ewes, we sought to determine whether this ionic peculiarity might explain the lack of responsiveness to Ca^{2+} influx. First, erythrocytes from both high K^+ and low K^+ sheep were exposed to ionophore and Ca^{2+}. All parameters shown in Table I were measured and neither cell type responded detectably Second, we suspended high K^+ and low K^+ sheep erythrocytes in both isotonic NaCl and isotonic KCl and, again, observed no response to calcium influx (results not shown) These two experiments indicate that the inherited variation in monovalent cation content of sheep erythrocytes is not directly responsible for the lack of response to Ca^{2+} influx.

Because red cells from adult sheep hydrolyze little intracellular ATP when exposed to A23187 and Ca^{2+}, we measured the Ca^{2+} ATPase activity of crude lysates of both human and adult sheep erythrocytes. Human erythrocytes display a very active Ca^{2+} ATPase in such preparations, consuming almost as much ATP as do whole cells under similar conditions (Table II). However, lysates of adult sheep erythrocytes have very little Ca^{2+} ATPase activity, in agreement with the lack of Ca^{2+}-induced ATP hydrolysis by whole sheep erythrocytes (Tables I and II). The stability of ATP in sheep erythrocytes incubated with A23187 and Ca^{2+}, therefore, appears to be due to a congenital absence of

TABLE II.[a]

	n	ATP hydrolyzed (μM/g Hb/hour)		
		+EGTA	+Ca^{2+}	Ca^{2+} ATPase Activity
nan	7	1.95 (±0.54)	5.04 (±0.46)	3.09 (±0.82)
ılt Sheep	5	2.86 (±0.61)	2.97 (±0.58)	0.11 (±0.18)
vborn Lambs	10	3.35 (±1.06)	6.54 (±1.13)	3.19 (±0.90)

ATPase activity of crude lysates of human, adult sheep and newborn (24-hr old) lamb red cells. Values in ses represent ±1 standard deviation. Ca^{2+} ATPase activity is expressed as μM ATP hydrolyzed/g Hb/hour lt of the addition of Ca^{2+} to the assay mixture. It represents the value obtained by subtraction of the ATP is in Ca^{2+}-free samples (+EGTA) from the ATP hydrolysis in Ca^{2+}-containing samples (+Ca^{2+}).

ve membrane Ca^{2+} efflux ATPase.

ontrast to erythrocytes from adult those from all newborn lambs have vels of intracellular K^+ and have an Na^+-K^+ ATPase, regardless of the ul- red cell K^+ phenotype of the offspring). We therefore investigated the Ca^{2+} ıiveness of red cells drawn from lambs ın 24 hr old. Surprisingly, we found wborn lamb red cells respond to Ca^{2+} precisely as do human red cells. That cells from newborn lambs shrink, shape, and hydrolyze large amounts ıcellular ATP (Fig. 1E and 1F, Table ates of lamb erythrocytes also have a ctive Ca^{2+} ATPase, similar to that in normal human red cells (Table II). ıight be expected from the presence rtain number of adult red cells, eryth- from newborn lambs are mosaic in sponse to Ca^{2+} influx; approximately of these cells shrink and become echinocytes, whereas 20–30% are mor- ically unaffected (Fig. 1F). The frac- Ca^{2+}-responsive cells gradually de- ıs the lamb matures, and all red cells month-old lambs are morphologically ted by Ca^{2+} influx. It appears that, in t to red cells from adult sheep, those ıewborn lambs have a multitude of characteristics, including increased $^+$ ATPase activity, fetal hemoglobin we have shown here, very active Ca^{2+} e and Ca^{2+}-responsive red cell mem- All these properties appear to change ert as the animal matures.

ussion. The lack of responsiveness of heep erythrocytes to intracellular Ca^{2+} s at least two puzzling questions. First, hard-put to explain the maintenance levels of intracellular Ca^{2+} *in vivo* in e of an almost total lack of membrane Ca^{2+} efflux ATPase activity. This may be explained either by an impermeability of the sheep red cell to external Ca^{2+} or by the presence of a membrane Ca^{2+} ATPase which has a very low V_{max} but a high Km for Ca^{2+}. Equally puzzling is the fact that polyacrylamide gel electrophoresis of membranes from adult sheep erythrocytes reveals the presence of proteins similar to both spectrin and actin. These two proteins are thought to be the major Ca^{2+}-responsive proteins in the erythrocyte membrane (20–22). Indeed, in irreversibly sickled cells from patients with sickle cell disease, these two proteins form a meshwork, insoluble in Triton X-100, which retains the shape of the intact cell (23). However, adult sheep erythrocytes, despite the presence of these two major membrane proteins, do not respond to Ca^{2+} influx. This indicates that other proteins, not yet identified, may be a necessary part of the Ca^{2+}-reactive apparatus of the erythrocyte membrane.

The dichotomous responses of adult sheep and newborn lamb red cells to intracellular Ca^{2+} may represent a promising model for the further elucidation of the mechanisms of Ca^{2+}-induced damage to red cells and of the ways in which erythrocytes respond, metabolically and morphologically, to increased intracellular Ca^{2+}.

Summary. Normal mammalian erythrocytes contain very small amounts of Ca^{2+}. When erythrocytes from most species of mammal are subjected to ionophore-induced Ca^{2+} accumulation, extensive morphologic and metabolic damage occurs. This is not true of red cells from adult sheep; these cells appear to be totally unresponsive to sudden increases in intracellular Ca^{2+}. In contrast, red cells from new born lambs are responsive to Ca^{2+} influx and gradually become unre-

sponsive during maturation of the animal. Although the precise reasons for the lack of response of adult sheep erythrocytes to Ca^{2+} accumulation is unknown, this system may provide a valuable model for the dissection of the various effects of Ca^{2+} upon cells and cell membranes.

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Effect of the Prolonged Immunization with Tumor-Unrelated Antigen on the Tumor Growth[1] (40087)

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Sera from tumor-bearing men and animals contain immune complexes (1, 2). It was shown that immune complexes in the sera from tumor-bearing animals are composed of the tumor antigen and its corresponding antibody (2). These sera may block cytotoxic activity of lymphocytes *in vitro* (for references, see 3) and enhance tumor growth (4). One could imagine that immune complexes from sera of the tumor bearers may specifically bind to lymphocytes or nonspecifically attach to Fc receptors of killer lymphocytes and inactivate them. These complexes may also attach to tumor cells in the same manner and mask tumor specific transplantation antigen. This raises the question whether tumor-unrelated immune complexes can attach to Fc receptors of the killer lymphocytes or tumor cells and inhibit anti-tumor response of the host. If this were the case, enhancement of tumor growth in the host with high level of the circulating immune complexes should be expected.

Formation of immune complexes in circulation and their deposition in the kidneys have been observed in rabbits (5) and mice (6) injected with antigen for a period of several months. In the present study, tumor growth was investigated in mice immunized for 4–5 months with tumor-unrelated antigens.

Materials and methods. Animals. C3H/HeHa mice, 12 weeks old, from West Seneca Laboratories, West Seneca, N.Y., were used in this study. The immunized and control animals were without any clinical signs of infection or sickness during the experimental period. Death of 30% of the immunized mice was due to the anaphylactic shock.

Tumor tissue. Methylcholanthrene-induced sarcoma, MCSa-22 was originally produced in this laboratory by subcutaneous injection of the carcinogen into a C3H/HeHa mouse. This tumor was maintained by transplantation in the strain of origin. Tumor tissue collected within 2–3 weeks after transplantation into normal mice was used in hemadsorption tests and/or for preparation of the tumor cell suspensions. Neoplastic tissue was minced, trypsinized and washed. Then suspensions were prepared that contained 10^5, 10^4, 10^3 or 10^2 cells in 0.2 ml of TC-199 medium supplemented with 0.02 *M* L-glutamine, 100 U of penicillin and 100 mg streptomycin/ml. These tumor cells were injected subcutaneously into experimental (immunized) and control (unimmunized) mice. Cryostat sections of tumors collected from immunized and control mice were tested for hemadsorption (see below).

Immunization and immune sera. Mice from one group were immunized daily for 4 to 5 months with bovine serum albumin (BSA) (Miles Laboratories, Inc., Elkhart, IN). The antigen was injected intravenously (iv) into the caudal tail vein at the dosage of 0.2 mg in 0.2 ml of phosphate buffered saline (PBS). Mice from another group were not immunized and served as a control. Mice from both groups were trial bled at the end of the third month of the immunization before injection with tumor cells and were terminally bled in the 4th and 5th month of the experimental period.

In another experiment, mice were injected intraperitoneally (ip) with ovalbumin (OA) (Sigma Chemical Company, St. Louis, MO). Here, again, daily injections of 0.2 mg of antigen in 0.2 ml of PBS were administered. The immune mice and corresponding control mice were bled out at the end of the 4th month of the experimental period.

All sera were stored at −70° and thawed quickly before testing. These sera were tested for circulating immune complexes and for inhibition of hemadsorption (see below).

Hemadsorption and inhibition of hemad-

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sorption. Hemadsorption by cryostatcut tissue sections was performed as previously described (7). Briefly, 10 μm thick tissue sections were placed on coverslips and dried at room temperature. Wells of microculture slides were filled with a suspension of sheep erythrocytes sensitized by a selected subagglutinating dilution of a rabbit anti-sheep erythrocyte serum. The wells were sealed with the coverslips in such a way that the tissue section was submerged in the suspension of sensitized erythrocytes. The preparations were incubated coverslip down for 1 hr at room temperature. Then they were turned coverslip up and left in this position at room temperature to allow unattached erythrocytes to fall down from the tissue. The sections were examined microscopically at low magnification. Adherence of erythrocytes to the sections was recorded as a positive hemadsorption test. If a tissue section was completely covered by erythrocytes, the test was graded as 4+. A reaction was considered 2+ if approximately half the tissue was covered by erythrocytes. Sparsely attached erythrocytes on the tissues were considered as a 1+ reaction. Absence of erythrocytes from the tissue was recorded as a negative result.

Inhibition studies were performed by incubating tissue sections with various sera for 20 min in a moist chamber at room temperature (8). Subsequently, the tissue sections were washed three times in PBS and the hemadsorption test was performed as described above. The lowest concentration of rabbit anti-sheep erythrocyte serum used for sensitization of sheep erythrocytes which showed 4+ hemadsorption was determined for each tumor specimen and employed in the inhibition studies.

Detection of immune complexes. Immune complexes in circulation were detected by using the Raji cell test (9) in a modification recently described (10). Briefly, a suspension of Raji cells (kindly supplied by Dr. T. Flanagan of the Department of Microbiology, SUNY at Buffalo, Buffalo, N.Y.) were harvested 72 hr after transfer. Fifty μl of Raji cells in RPMI-1640 medium (Associated Biomedic Systems, Inc., Buffalo, N.Y.) at a concentration of 5×10^6 cells/ml were incubated at 4° for 30 min with 50 μl of the tested serum or one of its dilutions. Subsequently, cells were washed with RPMI medium and stained at 4° for 30 min with 25 μl of fluorescein isothiocyanate (FITC)-labeled rabbit anti-murine IgG serum, with 25 μl of FITC-labeled rabbit anti-BSA serum, or with 25 μl of FITC-labeled rabbit anti-OA serum. Cells were then washed twice in RPMI medium and twice in PBS. One drop of the suspension was placed on a microscope slide and dried under a fan. Raji cells, incubated in the medium and then stained with FITC-conjugated sera, served as a control. Immune complexes in the cryostat-cut sections of the kidney were detected by staining with the same FITC-conjugated rabbit antisera.

Experimental design. At the time when immune complexes were detected in the kidneys from randomly selected immunized animals (approximately at 3 months of the immunization), all experimental and corresponding control mice were divided into groups and injected with 10^5, 10^4, 10^3 or 10^2 tumor cells. Cells from the 22nd passage of the syngenic tumor were injected into OA-immunized and corresponding control mice, whereas BSA immunized mice and corresponding control mice were injected with the cells from the 33rd passage of the same transplantable tumor. Subsequently, growth of the tumor was inspected daily. At the end of the experiment, all animals were bled out and the tumor was excised and weighed.

Statistical analysis. Data were analyzed by student's *t* test.

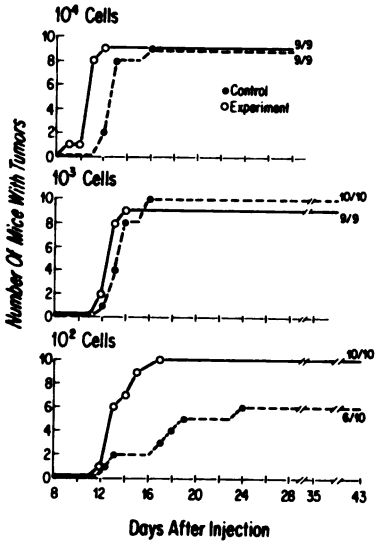
Results. Rate of tumor growth. At the time when deposits of immune complexes in the kidneys and circulating immune complexes in the sera of BSA-immunized mice could be demonstrated, the mice were divided into three groups and injected with 10^4, 10^3 and 10^2 tumor cells, respectively. Three groups of control mice were injected with similar numbers of the tumor cells. Figure 1 shows the results of this experiment. In all immunized and control mice injected with 10^4 and 10 tumor cells, tumors developed in 8–17 days after injection. All immunized mice injected with 10^2 tumor cells developed tumors at the same time. However, only three of ten control mice injected with 10^2 tumor cells developed tumors at that time. Three additional mice of this group developed tumors within 43 days.

Figure 2 shows the effect of prolonged

ıunization with OA on the rate of tumor
ɯth. When immune complexes appeared
he kidneys of OA-injected mice, immu-
:d and control mice were divided into
:e groups and injected with 10^5, 10^4 and
tumor cells. Tumors were detectable on
12th day after injection in all mice except
control mice injected with 10^3 tumor cells.
his group, tumor developed only in six of
mice and the remainder did not change
ing 21 days of observation.

'umor weight. In the experiments with
\ immunization, the tumors from mice
cted with 10^4, 10^3 and 10^2 cells were ex-
d and individually weighed on the 28th,
ı and 43rd day after injection, respec-
tively. Table I demonstrates the effect of long-term immunization with BSA on the weight of tumor mass. In all groups, tumors from immunized mice weighed significantly more than tumors from control animals.

Tumors from OA-immunized mice and corresponding control mice were excised and weighed on the 21st day after injection with 10^5 and 10^3 tumor cells. Weight of the tumors from immunized mice injected with 10^5 tumor cells was significantly greater than that of control mice injected with the same number of tumor cells. Tumor weight did not significantly differ between immunized and control mice injected with 10^3 of the tumor



IG. 1. Tumor growth in BSA-immunized and con-
mice injected with 10^4, 10^3 and 10^2 syngenic tumor
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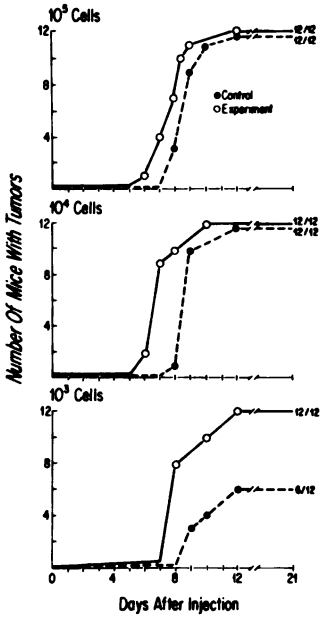

FIG. 2. Tumor growth in OA-immunized and control mice injected with 10^5, 10^4 and 10^3 syngenic tumor cells.

TABLE I. EFFECT OF PROLONGED IMMUNIZATION OF MICE WITH BSA OR OA ON THE WEIGHT OF THE TUMOR.

Mice	Weight of tumors (mg) from mice injected with tumor cells			
	10^5	10^4	10^3	10^2
Immunized[a] with BSA	ND[b]	1326 ± 336[c,e]	1438 ± 209[e]	1831 ± 354[e]
Control	ND	470 ± 72	433 ± 86	416 ± 140
Immunized[d] with OA	1235 ± 266[e]	ND	385 ± 108	ND
Control	448 ± 90	ND	272 ± 102	ND

Tumors from BSA-immunized and control mice injected with 10^4, 10^3, and 10^2 tumor cells were excised and ghed on the 28th, 35th and 43rd day after injection, respectively.
Not done.
Mean ± SD.
Tumors from OA-immunized and control mice injected with 10^5 and 10^3 tumor cells were excised and weighed he 21st day after injection.
Denotes statistically different from control at $P<0.05$.

cells (Table I).

Hemadsorption and inhibition of hemadsorption. Cryostat sections of tumors from mice immunized with BSA or OA and control mice did not show any difference in the adsorption of the sensitized sheep erythrocytes. Sera from tumor-bearing mice immunized with either BSA or OA markedly inhibited hemadsorption, whereas, sera from tumor-bearing non-immunized mice and from normal mice did not inhibit hemadsorption. Table II presents results of such inhibition experiments in which hemadsorption by tumor tissue and by normal spleen tissue was examined.

Immune complexes were deposited in kidneys of the mice after 2 to 3 months of the injections with BSA or OA. These complexes were stained with FITC-labeled rabbit anti-murine IgG. In some experiments they were stained with FITC-labeled rabbit anti-BSA or rabbit anti-OA, respectively. Before tumor cells were injected into the mice, the immune complexes were demonstrated in all of the kidneys taken from five randomly selected mice of the group of the animals immunized with BSA and five mice immunized with OA. In the kidneys collected when the tumors were harvested from the immunized mice with either BSA or OA, immune complexes were detected in 80% by staining with anti-BSA serum or anti-OA serum, respectively, and in 100% by staining with anti-murine IgG serum. Immune complexes in the kidneys taken from control (unimmunized) mice injected with tumor cells were not detected by using anti-BSA serum or anti-OA serum. However, they were detected in 40% of mice by staining with anti-murine IgG serum. A similar percentage of the positive staining was also noticed in the kidneys from normal mice. Deposition of IgG has previously been described in kidneys of aging mice (11).

TABLE II. ADSORPTION OF SENSITIZED SHEEP ERYTHROCYTES BY C3H TUMOR AND SPLEEN TISSUES. INHIBITION BY SERA FROM NORMAL MICE, TUMOR-BEARING UNIMMUNIZED MICE AND TUMOR-BEARING IMMUNIZED MICE.

Hemadsorption by:	Serum dilution 1 to:	Serum from normal mouse	Sera from tumor-bearing mice	
			Unimmunized	Immunized
Tumor tissue	10	4+	3+	—
	20	4+	4+	—
	40	4+	4+	2+
Spleen tissue	10	4+	3+	—
	20	4+	4+	1+
	40	4+	4+	1+

The same FITC-labeled reagents were used to demonstrate circulating immune complexes in sera of immunized mice by means of the Raji cell assay. Using anti-BSA serum, the average titer of the circulating immune complexes was 1:8 ranging from 1:2 to 1:32 in the individual sera from trial bleeding of BSA-immunized mice. This titer decreased to 1:2 ranging from 1:1 to 1:4 in the sera from terminal bleeding of the BSA-immunized mice. In the sera from terminal bleeding of OA-immunized mice, the circulating complexes were detected in the average titer of 1:4 ranging from 1:2 to 1:16. OA anti-OA complexes were not tested in the sera from trial bleeding of OA-immunized mice. Immune complexes composed of BSA or OA and their corresponding antibodies were not detectable in the sera from control mice injected with tumor cells or from normal mice.

Using FITC-labeled rabbit anti-murine IgG serum in Raji cell assay with sera from trial bleeding of BSA-immunized mice, circulating immune complexes were in the average titer of 1:32 ranging from 1:8 to 1:64. A similar titer of circulating immune complexes was observed in the sera from the terminal bleeding of BSA- or OA-immunized tumor-bearing mice and control mice injected with tumor cells. Interestingly, the circulating immune complexes were also detected in the sera from normal mice at the average titer of 1:8 ranging from 1:4 to 1:32. The increased amount of circulating immune complexes in control mice injected with tumor cells was possibly due to tumor growth.

Discussion. The rate of tumor growth in mice immunized with BSA and injected with 10^2 tumor cells and in mice immunized with OA and injected with 10^3 tumor cells was markedly greater than that in control non-immunized mice (Figs. 1 and 2). Tumor weight was also significantly greater in mice immunized with BSA and OA than nonimmunized mice (Table I). Similar enhancement of tumor growth was noted in immunologically impaired mice injected with a mixture of the tumor cells and sensitized lymphocytes (12, 13). Jeejeebhoy (14) has reported that a certain population of the sen-

ymphocytes is responsible for en-
nt and another for inhibition of tu-
wth. Lymphocytes collected on the
after injection of tumor cells into
ıanced growth in the tumor *in vitro*,
lymphocytes collected by day 12 in-
ɔrmation of the neoplastic cell colo-
ɛnt observations suggested that im-
nulation of tumor growth *in vitro* is
l by T and B cells that are distinct
ɔtoxic effector cells (15).
ata of this study demonstrated that
ontrol mice injected with 10^3 tumor
'eloped tumors in one experiment
whereas in another experiment per-
months later, the tumors developed
ɔf the control mice injected with the
ɜe of the same tumor (Fig. 1). This
could be due to the changes in the
: of this tumor with the progressing
ɔf the passages. Another possibility
ɛells from the tumor used in one
ɪnt contained relatively more mac-
than the cells from the tumor em-
ı another experiment. The number
ɔphages in the inoculum may ob-
ıave influence on the tumor growth
'ed by Wood and Gillespie (16).
study, mice were immunized with
ırelated antigens for 4–5 months. In
:hs, deposition of immune complexes
d of BSA or OA and corresponding
ɜs in kidneys was observed. The im-
ɪmplexes were detected in sera of
ɔe by Raji cell assay and by inhibi-
.emadsorption test. Other investiga-
ɛrved deposition of immune com-
ı kidneys of F1 hybrid mice with
ɜus-host reaction resulting from
ıtation of parental lymphoid cells
was suggested that antigens of the
ɪrmed immune complexes with their
ıding antibodies produced by the
cells and that these complexes were
l in the kidneys (18). Furthermore,
us antigenic stimulation of the im-
stem in animals with graft-versus-
ɪtion lead to an increased incidence
ısia (19). It is quite possible that in
/, daily injection of an antigen con-
' stimulated lymphocytes which, in
ld contribute to the enhancement of
r growth.

Daily injection of antigens could also result in continuous formation of circulating immune complexes which could attach to Fc receptors of the cytotoxic lymphocytes and inactivate these cells (20, 21). A recent study indicated that most mouse cytotoxic lymphocytes possess Fc receptors (22). In addition, circulating immune complexes could attach to the phagocytic cells and decrease their ability to penetrate into the site of tumor cell injection (23). A decreased number of phagocytic cells at the tumor site might have affected tumorcidal activity, attraction of lymphoid cells into tumor site and the interaction between macrophages and lymphocytes which, in turn, might have diminished immune response to the tumor antigen.

Alternatively, circulating immune complexes could also attach to the tumor cells and mask tumor specific transplantation antigens; therefore, the immune system of the host could not recognize them. This suggestion is supported by the observation that immune complexes can attach to the Fc receptors of tumor tissue (24) or tumor cells (25).

Summary. The effect of prolonged immunization of mice with bovine serum albumin (BSA) or ovalbumin (OA) on the growth of the transplanted, methylcholanthrene-induced fibrosarcoma was studied. When immune complexes appeared in the kidneys and in the circulation, mice were injected subcutaneously with various numbers of syngeneic tumor cells.

Tumor developed within 17 days in all mice immunized with BSA and injected with 10^4, 10^3 and 10^2 tumor cells and control mice injected with 10^4 and 10^3, whereas only 3 of 10 control mice injected with 10^2 tumor cells developed tumors at that time. All mice immunized with OA and injected with 10^5, 10^4 and 10^3 tumor cells and control mice injected with 10^5 and 10^4 developed tumor within 12 days after injection of the tumor cells, whereas in the control group injected with 10^3 tumor cells, only 6 of 12 mice did so. Weight of the tumors from the mice immunized with BSA or with OA was significantly greater than that of tumors from control mice.

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'romoting and Inhibitory Activities Of 3T3 and Other Cell Lines for Thiol-dependent Lymphoma Cells *in Vitro (40088).*

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riety of growth factors exist for
ls in culture, and their study may
an understanding of mecha-
sible for growth control *in vivo*
thiols and disulfides have an
tion in this regard because al-
profoundly modify the survival
growth of mouse cells *in vitro*
er nonphysiological substances
red in the medium in much
physiological concentrations (2).
it experiments have been per-
udy conditions *in vitro* which
the provision of thiols unneces-
ve used Lymphoma L1210(V),
ighly thiol dependent and
hiol independent variant (2, 3).
now that 3T3, L and other cell
pport the growth of L1210(V)
sence of thiol. The macrophage,
athan and Terry does not there-
spect have a unique relationship
id cells (4). Cocultivation failed
permanent conditioning of the
nonetheless we demonstrate that
otion of the lymphoma cells is
norally.

spect of this system is shown by
nes which support lymphoma
cell density but inhibit growth
cultures.

and methods. Reagents. 2-Mer-
(2-ME) and Concanavalin A
obtained from Sigma Chemical
. Louis, MO.

edium. Dulbecco's modification
edium (DMEM) was used with
CO2 atmosphere (2). It was sup-
ith 10% horse serum (Ho.S).

Normal $B_6D_2F_1$, male, 18–20 g,
ld were obtained from the Jack-
ries, Bar Harbor, ME.

10(V) and L1210(A) (2, 3) were
as ascites tumors in B6D2F1
a mineral oil induced malignant
histiocyte tumor was kindly provided by Dr. Henry Azar. Mouse L cells were obtained from Dr. Karl Lanks. 3T3 Cells of various sublines were used. First were Balb/c cells obtained by Dr. Michael Howe from Microbiological Associates, Bethesda and carried in his laboratory for one year by splitting at intervals of 3 or more days. In the course of this time the cells lost contact inhibition and are considered "spontaneously transformed." Examination for mycoplasma sp. by Microbiological Associates, Diagnostic Labs. was negative. Other sublines, namely, Swiss 3T3 (ME) and Balb/c 3T3 were obtained from Dr. Ishwari Prasad.

Millipore diffusion chamber culture. Chambers were constructed and prepared as described previously and sterilized by ethylene oxide (5). All chambers were put up with 10^6 L1210(V) cells. They were placed on glass capillary pipettes (Microcaps, 100 microliters, Drummond Scientific Company, Broomall, PA), which were anchored with dental wax, 1 cm apart, on the bottoms of 60 × 15 mm Falcon tissue culture dishes (Becton, Dickinson and Co., Oxnard, CA) and immersed in culture medium.

Cocultivation of L1210(V) cells. (a) *With established tissue culture lines.* Supporting cells (3T3, etc.) were suspended after treatment with 0.25% trypsin, washed in saline, and inoculated into T flasks (Falcon, 25^2 cm) at densities of 1×10^5 cells and above. 3T3 Cells, 2×10^6, produced a confluent monolayer on attachment. The medium was changed 16–18 hr later, 1×10^6 lymphoma cells were added, and the cultures incubated for a further 48 hr. Lymphoma cells were then suspended by shaking and counted. They were easily distinguishable by their size, rounded shape and the presence of refractile cytoplasmic granules.

(b) *With embryonic cells.* Eight millimeter BALB/c mouse embryos were fragmented by cutting and agitation and trypsinized as

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above. Single cell suspensions were obtained by sedimentation. Monolayer cultures were then prepared and subcultured three times before use with lymphoma cells.

(c) *With macrophages.* Aliquots of $B_6D_2F_1$, nucleated spleen cells (2×10^7–2×10^8 in each experiment) were inoculated as described above in 4.5 ml medium. After 24 hours the flask surface was washed and the medium replaced. The density of adherent cells in all flasks of an experiment was then checked for uniformity by counts of the average number of cells in five high power fields; in no case was variation more than 10%. The absolute number of cells in one flask was determined by counting the suspension produced by thorough scraping with a rubber tipped rod. Adherent cell counts in different experiments varied from 5×10^5 to 5×10^6, and 98% of cells phagocytized carbon (four experiments). After these determinations were made, 5×10^6 L1210(V) cells were added and they were recounted 48 hours later.

Similar procedures were used with peritoneal macrophages.

Results. All standard tissue culture cell lines tested and also mouse embryo cells were able to support the growth of L1210(V) cells (Fig. 1).

3T3 Cells of different sublines were effective, irrespective of whether they showed contact inhibition or were transformed. Inocula of 5×10^5 3T3 cells per culture were optimal and 1-2 divisions of the lymphoma cells occurred within 48 hr.

When L1210(V) cells were stimulated to vigorous growth, addition of 2-ME increased the rate of lymphoma cell division only minimally. Thus, 2.00 ± 0.03 divisions occurred in 48 hr in the presence of 10^6 SV-3T3 (ME) cells. With added 2-ME, the corresponding figure was 2.25 ± 0.02.

In a single case, that of a BALB/c 3T3 subline which had lost contact inhibition during continuous passage ("spontaneous transformation") there was a notable dependence on population density. Two hundred thousand of these cells per culture failed to promote growth of lymphoma cells. At 4×10^5 cells, however, one division was produced but 2×10^6 cells were again ineffective. However, although no division of lymphoma cells occurred, with this inoculum more than 90% of the latter cells were viable at 48 hours. Strain L cells showed a similar density relationship the optimal density was 1×10^5–2×10^6 cells per culture, and over this figure growth promotion diminished.

These results were compared to other findings with macrophage cultures (Fig. 2), which have previously been shown to support the growth of mouse lymphoid cells (4, 6). At inocula of 5×10^5 feeder cells, macrophages produced approximately the same degree of growth promotion of lymphoma cells (1.0–1.5 divisions) as did the other cell lines tested. But as the cell density of the macrophages increased, lymphoma cell growth became increasingly rapid, up to a level of 2.8 divisions at 2×10^6 cells. At this density, growth was at least equal to that produced by optimal concentrations of 2-ME (2.28 ± 0.14 divisions). Macrophages of various sources and a histiocyte cell line (TP 5-9) derived from a tumor which followed mineral oil carcinogenesis were all strong growth promoters, but thioglycollate stimulated peritoneal cells at 2×10^6 per culture seemed the most effective of

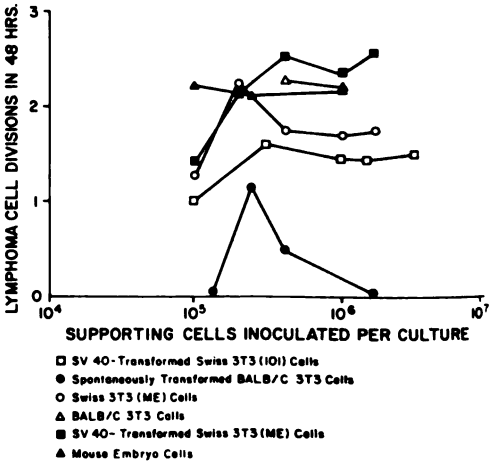
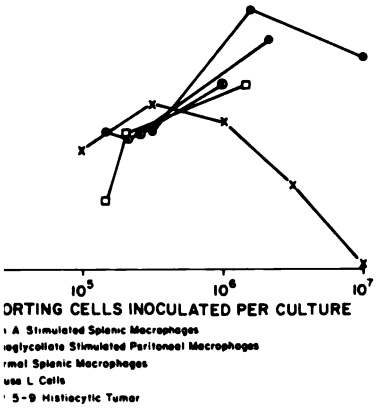


FIG. 1. Multiplication of L1210(V) lymphoma cells cocultivated with various cell lines in the absence of thiol compounds. Assays of growth promoting activity of feeder cells were performed in several experiments. In each of these controls were set up (a) negative-L1210(V) cultured alone in unmodified DMEM: no cell division occurred. (b) positive-L1210(V) cultured alone in DMEM + 5 μM 2-ME: 2.28 ± 0.14 cell divisions resulted. To ensure that the results obtained were not due to trypsin carried over by the feeder cells, this enzyme was added in concentrations of 0.001%–0.02% to culture media. L1210(V) cells inoculated into these in the absence of feeder cells failed to divide unless 2-ME was present.



ultiplication of L1210(V) lymphoma cells
vith macrophages from various sources and
absence of thiol compounds. Methods as in

pe examined.
e experiments, therefore, macro-
ve only growth promotion and not
. They were therefore chosen for
periments to examine growth pro-
iechanisms, and "spontaneously
ed" BALB/c 3T3 at high density
ory effects.

ism of growth promotion. Superna-
: removed from 24, 48 and 72 cul-
acrophages (5×10^5 to 1×10^7 cells)
l been shown to provide optimal
L1210(V), and they were inocu-
lymphoma cells (1×10^6/ml). After
iphoma cell recovery was 45% to
ie inoculum, in 12 experiments, but
nts were in no case able to sustain
n the other hand, when 5 μM 2 ME
to the supernatants, the lymphoma
iplied (1.6 ± 0.04 divisions in 48

ssibility that macrophages would
ition the medium in the presence of
was then examined. It was ob-
it once mixed cultures of macro-
d lymphoma cells were established
stently supported lymphoma cell
ig. 3). The macrophages remained
/hile the lymphoma cells in suspen-
ferated. At 48 hour intervals, part
:dium with suspended lymphoma
removed and fresh medium was
)ring the lymphoma cell density to
he original inoculum (1×10^6

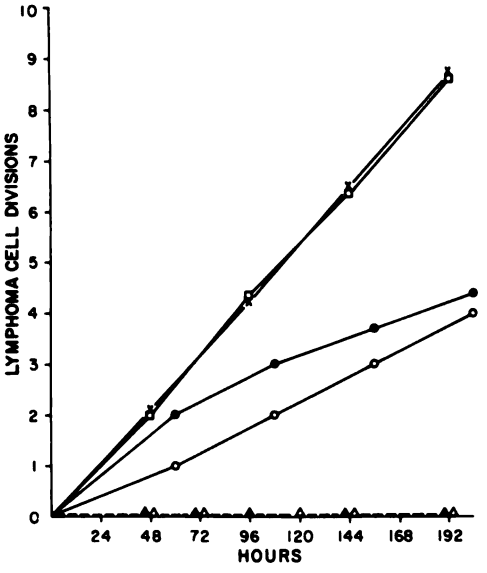


FIG. 3. Cumulative divisions of L1210(V) cells, under various conditions. ×, 5 μ*M* 2-ME, no feeder cell. ▲, No thiol, no feeder cell. □, No thiol, thioglycollate-stimulated peritoneal macrophages (2×10^6 per culture). ●, No thiol, Con A-stimulated splenic macrophages (2×10^5 per culture). ○, No thiol, normal splenic macrophages (2×10^6 per culture). △, No thiol, L1210(V) cells grown for 48 hr with thioglycollate-stimulated peritoneal macrophages, then placed in new flask without feeder layer.

cells/ml). On removing the medium with suspended cells 48 hr after changing and transferring to a fresh flask, no increase in cell number occurred, unless, as before, thiol was added (Fig. 3).

These experiments made it necessary to determine whether direct contact between lymphoma cells and macrophages or other cells was necessary for growth and this was examined by the use of diffusion chambers.

Diffusion chamber experiments. Petri dishes were plated with macrophage or other cell suspensions as in the preceding experiments, after 48 hr the medium was replaced and diffusion chambers containing known numbers of lymphoma cells were inserted. The chambers were positioned about 1.5 mm above the feeder cell layer.

When the diffusion chambers were removed after 72 hr, it was seen that macrophages had caused proliferation of the lymphoma cells (1.3–2.6 divisions); SV40 transformed 3T3 cells were also effective, but in the experiment performed less so than mac-

rophages (1.4 versus 2.6 divisions). With both cell types viability of the lymphoma cells was markedly increased. (Table I).

Again, supernatants removed from the macrophage culture before or after addition of the lymphoma cells failed to support growth unless thiol was added.

With other experimental work using diffusion chambers, objections have been raised that projections of cell processes through the pores of the membranes could be responsible for the effects observed (7). In the present experiments not only was fine porosity membrane used (0.1 μm), but the membrane was 150 μm thick and it was more than 1 mm from the macrophage layer.

These experiments indicate therefore that the feeder cells can affect lymphoma cell growth through humoral factors.

Cause of the failure of growth promotion by "spontaneously transformed" BALB/c 3T3 cells at high density. To test whether the failure of these cells at high density to support the growth of L1210(V) cells was related to or separate from their thiol replacing role, a subline, L1210(A) which is thiol independent *in vitro* was examined. Like L1210(V) this subline completely failed to grow in contact with 3T3 cells at high density nor did it do so in supernatant medium (Fig. 4). This observation and the inability of added thiol (to 50 μm 2-ME) to restore growth indicated that growth failure in this supernatant was distinct from thiol dependency.

Dilution of supernatant with an equal volume of fresh serum-containing medium permitted some growth (1.6 cell divisions in 48 hr compared with 2.5 cell divisions in com-

TABLE I. GROWTH PROMOTION BY SPLENIC MACROPHAGES SEPARATED FROM LYMPHOMA CELLS BY THE MEMBRANE OF A DIFFUSION CHAMBER.

	Cell type on Petri dish	2-ME (μM)	L1210(V) cells inoculated into diffusion chamber (×10⁶)	L1210(V) cells recovered at 72 hr (×10⁶)			
				Viable[a]	Dead	Total	Cell divisions
Expt. 1	None	0	1.0	0.2	0.3	0.5	0.0
	None	5	1.0	5.6	0.5	6.1	2.5
	1.3×10^6 splenic macrophages	0	1.0	2.4	0.1	2.5	1.3
Expt. 2	None	0	1.0	0.2	1.4	1.6	0.6
	None	5	1.0	6.2	0.6	6.8	2.7
	4×10^6 peritoneal macrophages	0	1.0	6.0	0.3	6.3	2.6
	5×10^6 SV40-transformed 3T3 (ME) cells	0	1.0	2.7	0.2	2.9	1.5

[a] Viability determined by trypan blue exclusion. Peritoneal macrophages were obtained from BDF mice injected with 2 ml thioglycollate medium 4 days previously.

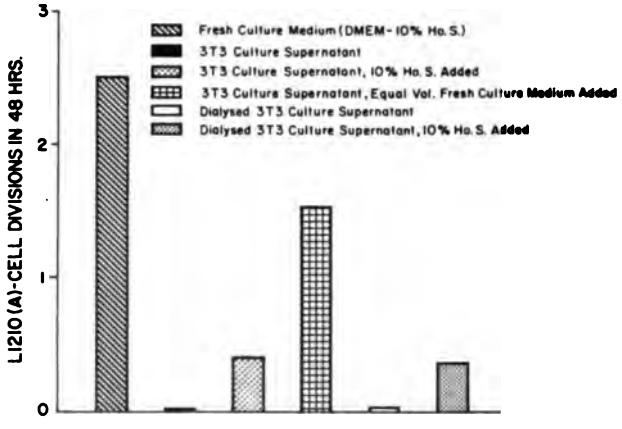

Fresh Culture Medium (DMEM - 10% Ho. S.)
3T3 Culture Supernatant
3T3 Culture Supernatant, 10% Ho. S. Added
3T3 Culture Supernatant, Equal Vol. Fresh Culture Medium Added
Dialysed 3T3 Culture Supernatant
Dialysed 3T3 Culture Supernatant, 10% Ho. S. Added
L1210 (A)-CELL DIVISIONS IN 48 HRS.
3
2
1
0

FIG. 4. Inhibition of growth of L1210(A) cells by supernatants from "spontaneously transformed" BALB/c 3T3 cells. 3T3 cells were inoculated at (4×10^7 cells) in 60 ml and incubated for 48 hr. Supernatant was removed at this time and aliquots were treated as shown, 1×10^6 L1210(A) cells/ml were then added and recounted after 48 hr. Dialysis was carried out against 100 volumes of fresh DMEM without serum.

resh medium). On the other hand, of supernatant against fresh Dulnedium had no effect in restoring the of the medium to support growth. rum added to such medium had only growth promoting effect (0.4 cell in 48 hr). The latter two experiments that in the present context, depletion nts in the medium by 3T3 cells is of y minor importance in preventing na growth. It appears likely therefore ondialyzable inhibitor of growth of homa cells is produced by 3T3. This vaits further characterization.

sion. The experiments described at a variety of cells, in addition to the age, can produce conditions in culich make the provision of growth ng thiols and disulfides unnecessary 10(V) cells (Table I, Fig. 1–3). That factors are involved is apparent from sion chamber experiments (Table I), prisingly macrophages and other ells are unable to condition the mermanently.

sible explanation is that growth profactor(s) from the feeder cells are and must be continuously produced ivated. There are however, several ossibilities; the concentration of promoting factor in the external meight limit its production by a tight k between utilization and production: ively feeder cells may be necessary nactivation of soluble autoinhibitory s of the lymphoma cells. Further exts are required to permit a decision these different mechanisms.

experiments now described have another aspect of growth control isms for lymphoma cells by the inhibtion of higher cell densities of certain L cells, which appears to be meit least in the former case by a nonle product(s). The putative inhibitor e may have either a high molecular or it may bind to protein in the meimilar inhibition has been reported tor(s) in supernatants of macrophage of considerably higher density than those now used (8).

The possibility that the growth inhibition now observed was due to arginine deprivation as a result of contamination of 3T3 cells with mycoplasma was excluded by direct measurement using a Jeolco JLC-5AH analyzer; less than 30% of the initial arginine content of the medium was metabolized under the conditions of the experiments (9).

The significance of factors such as those now described for L1210 cells *in vitro* to growth control systems *in vivo* is uncertain. Clearly however, it is only by the use of simple *in vitro* systems that growth factors can be characterized and isolated. They will then be available for studies in systems of increasing complexity and finally in the whole animal.

Summary. The growth of thiol dependent cells of Lymphoma L1210(V) is supported by 3T3 and L cells as well as by macrophages *in vitro.* The supporting cells do not permanently condition the medium, but diffusion chamber studies show that growth promotion is mediated humorally. At high cell density inhibition of lymphoma cell growth can be observed in the supernatant from one of the 3T3 sublines examined, which appears to be due to a nondialyzable inhibitor whose effect is unrelated to thiol dependency.

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CONTENTS

SCIENTIFIC PROCEEDINGS, VOUME 157

Six hundred seventy-eighth issue, January 1978 1

Six hundred seventy-ninth issue, February 1978 159

Six hundred eightieth issue, March 1978 337

Six hundred eighty-first issue, April 1978 523

Author Index 697

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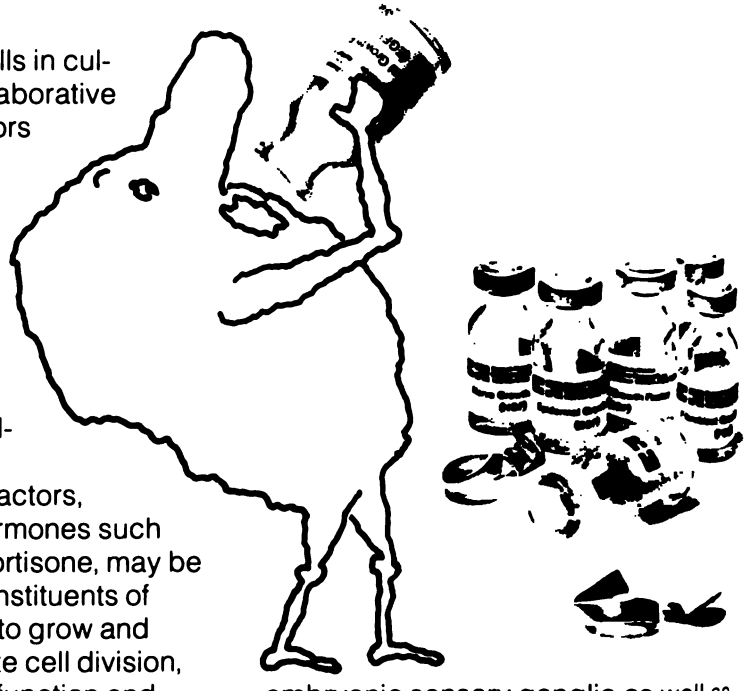

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biochem-Behring i

ırles River ..... Cover 4

laborative Research viii

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TABLE OF CONTENTS

BIOCHEMISTRY

Calcium–Phospholipid–Phosphate Complexes in Mineralizing Tissue	A. L. BOSKEY, M. R. GOLDBERG, A. S. POSNER	590
Sparsomycin-Induced Disaggregation but not Detachment of Hepatic Membrane-Bound Polyribosomes	H. SIDRANSKY, E. VERNEY, C. N. MURTY, D. S. R. SARMA	660
Unscheduled DNA Synthesis in Rat Lens Epithelial Cells Induced by Alkylating Agents	J. G. JOSE, K. L. YIELDING	688

ENDOCRINOLOGY

Inhibition of Bovidae Oocyte Meiosis by Serine Protease Inhibitors	G. JAGIELLO, M. DUCAYEN, W. GOONAN, S. DOWNEY	550
Effects of Dopamine, Norepinephrine, and Serotonin on Plasma Prolactin Levels in Ovariectomized, Pituitary-Grafted Rats	J. I. LEVIN, J. L. VOOGT	576
Estimation of Plasma Half-Lives of Vasopressin Analogs from Vasopressor Responses by Curve-Fitting	D. GAZIS, W. H. SAWYER	584
An Immunological Comparison Between Mouse α-Fetoprotein and the Uterine Estrogen Receptor	D. O. TOFT, T. B. TOMASI, JR.	594
Prolactin Secretion from Clonal Pituitary Cells following Incubation with Estradiol, Progesterone, Thyrotrophin Releasing Hormone and Dopamine	D. C. HERBERT, H. ISHIKAWA, M. SHIINO, E. G. RENNELS	605
Inhibition of Insulin and Glucagon Secretion by Somatostatin: are There Indirect Effects?	P. H. SMITH, M. CHEN, S. C. WOODS, J. W. ENSINCK, D. PORTE, JR.	643
Serum Triiodothyronine Concentrations in Riboflavin-Deficient, Diabetic and Normal Mice	A. S. REDDI	690

GROWTH AND DEVELOPMENT

Induction of Malformations by Actinomycin D at Mid-Pregnancy in the Rat and Protection by L-Thyroxine	J. E. HARVEY, H. H. SREBNIK	553
The Influence of Rat Age on Crypt Cell Kinetics after Partial Resection of the Small Intestine	F. R. KIRCHNER, J. W. BYBEE, J. W. OSBORNE	572

HEMATOLOGY

Granulocyte Separation by Modified Centrifugal Elutriation System	M. D. PERSIDSKY, L. S. OLSON	599

IMMUNOLOGY

Differential Effects of Cholera Toxin Pre-treatment on *In Vitro* vs. *In Vivo* Immunocyte Responses	S. F. LYONS, H. FRIEDMAN	631
Blockade of Mitogen Induction of the Interferon Lymphokine by a Phenolic Food Additive Metabolite	D. L. ARCHER, H. M. JOHNSON	684

NUTRITION

Influence of Feeding Frequency on Body Weight and Glucose Tolerance in the Pig	D. R. ROMSOS, E. R. MILLER, G. A. LEVEILLE	528
Biological Evaluation of Crystalline Fraction I Protein from Tobacco	B. H. ERSHOFF, S. G. WILDMAN, P. KWANYUEN	626

ONCOLOGY

Characterization of Transplantable Myelomonocytic Leukemia WEHI-3B in Syngeneic BALB/c Mice	W. H. MURPHY, H. B. URNOVITZ, J. L. MARYANSKI, G. D. ABRAMS	556

The Effect of Bran on Dimethylhydrazine-Induced Colon Carcinogenesis in the Rat — T. A. BARBOLT, R. ABRAHAM ... 656

Malignant Conversion of Shope Papillomas and Associated Changes of Serum Ceruloplasmin in Domestic Rabbits — A. SETO, H. TOKUDA, Y. ITO ... 694

PATHOLOGICAL PHYSIOLOGY

Allopurinol-Induced Myocardial and Renal Damage in Non-arteriosclerotic (Virgin) and Arteriosclerotic (Breeder) Sprague-Dawley Rats — B. C. WEXLER, B. P. GREENBERG ... 541

Pulmonary Vascular Resistance and Right Ventricular Function in Canine Endotoxin Shock — W. G. GUNTHEROTH, I. KAWABORI, J. G. STEVENSON, N. R. CHOLVIN ... 610

Effects of Leukocytic Endogenous Mediator on Hemopexin, Transferrin, and Liver Catalase — C. R. MERRIMAN, H. F. UPCHURCH, R. F. KAMPSCHMIDT ... 669

Cigarette Smoking Baboon Model: Demonstration of Feasibility — H. C. MCGILL, JR., W. R. ROGERS, R. L. WILBUR, D. E. JOHNSON ... 672

PHARMACOLOGY

Hemodynamic Changes during Acute Salicylate Intoxication in Conscious Dogs — B. C. WANG, N. J. MUETING, K. L. GOETZ 531

Protective Action of Methylprednisolone on the Isolated Perfused Rat Heart Following Severe Hypoxia — R. W. BUSUTTIL, W. J. GEORGE ... 580

Indomethacin: Relationship between Ulcerogenic and Anti-Inflammatory Properties. I. Effects of an Intestinal Lesion-Preventing Fat-Free Diet on Anti-Edema and Anti-Granuloma Properties of Indomethacin in the Rat — G. VOLTERRA, O. DEL SOLDATO, A. MELI 615

Inhibition of Prostaglandin-Induced Pulmonary Vasoconstriction by Organic Acid Transport Inhibitors — T. C. WICKS, P. W. RAMWELL, P. A. KOT, J. C. ROSE ... 677

PHYSIOLOGY

Control of A-V Shunt and Capillary Circuits in the Dog Hindpaw — C. H. BAKER, D. L. DAVIS, E. T. SUTTON 536

Effects of Synthetic Vasoactive Intestinal Peptide (VIP), Secretin and Their Partial Sequences on Gastric Secretion — G. M. MAKHLOUF, A. M. ZFASS, S. I. SAID, M. SCHEBALIN ... 565

Tonic Sympathoinhibition in the Baroreceptor Denervated Cat — S. M. BARMAN, G. L. GEBBER ... 648

Renin and Aldosterone Levels in Dolphins and Sea Lions — R. L. MALVIN, S. H. RIDGWAY, L. CORNELL ... 665

Effects of Carbon Monoxide on Isolated Heart Muscle — J. J. MCGRATH, L. G. MARTIN ... 681

VIROLOGY

Effect of Immunization of Mothers on Cytomegalovirus Infection in Suckling Mice — D. N. MEDEARIS, JR., S. L. PROKAY ... 523

Effect of Heparin on Cytomegalovirus Replication — V. C. CHOI, N. S. SWACK, G. D. HSIUNG 569

N-Acetylneuraminic Acid (NANA) in Measles Virus — P. DORE-DUFFY, C. HOWE ... 622

Live Respiratory Syncytial Virus Vaccine Administered Parenterally — E. B. BUYNAK, R. E. WEIBEL, A. A. MCLEAN, M. R. HILLEMAN ... 636

is OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 523-527 (1978)

ct of Immunization of Mothers on Cytomegalovirus Infection in Suckling Mice[1] (40089)

DONALD N. MEDEARIS, JR.[2] AND SANDRA L. PROKAY

ent of Pediatrics, Case Western Reserve University and Cleveland Metropolitan General Hospital, Cleveland, Ohio 44109

duction. Human cytomegalovirus /) infections cause mental retardation deafness in an estimated 1% of the born in the U.S. (1). This morbidity is ly the result of an intrauterine HCMV ın of the brain (CNS) (2), and it might 'ented by immunization (3). Immuni-)f a woman protects her offspring from 's teratogenic effects (4); and Bram- eview of passive transfer of antibody ıother to offspring indicates that such r often confers protection to the young ıriety of species, including man and (5). Studies of mouse cytomegalovirus V) have provided information relevant erstanding HCMV infection (2), and ty in suckling mice due to MCMV luced by immunization of the mother conception (6).

efore, the studies to be reported were) determine whether (a) CNS infection ıe induced regularly by intraperitoneal ıoculation of newborn mice with /, (b) this infection and its attendant ity and mortality could be prevented ınunization of the weanling female o pregnancy, and (c) studies of the e response in this situation would re- fferences that might help us under- /hich response(s) conferred protection.

:rials and methods. Mice. Swiss Web- :e obtained from National Laboratory l Company (O'Fallon, MO) were used xperiments.

s. The Smith strain of MCMV was d by Dr. June Osborn, University of ısin, maintained by mouse passage, and prepared and stored essentially as described by her (7). Uninfected (normal) salivary gland suspensions were prepared in the same way from glands of uninoculated mice and employed as a control.

Cell culture and media. Primary mouse embryo cell cultures (MECC) prepared from 15- to 17-day old embryos, were grown in Medium (M) 199 (GIBCO) containing 10% heat inactivated calf serum (CS) (GIBCO) and maintained in M199 with 5% CS. Secondary MECC monolayers were prepared by seeding plastic tissue culture flasks (Falcon 25 cm^2) with 2×10^6 cells. All media contained antibiotics.

Viral assay. Tissues were assayed for MCMV plaques on secondary MECC flasks using 0.8% tragacanth in maintenance media as overlay. All points represent the results of testing a pool of organs from at least two animals, and all plaque assays were run in duplicate.

Tissues from some animals which had been inoculated ip as sucklings were also tested for virus by an explant method 60 or more days after birth. Organs were removed aseptically, pooled, minced in small amounts of media and extruded through needles into Falcon flasks. Salivary glands were minced finely and seeded into the flasks without forced extrusion. Minimal essential media (MEM) (GIBCO) with 20% fetal calf serum (FCS) was used to promote growth of these explant tissues. Cultures were observed for cytopathic effect (CPE) for 2 months during which they were also subcultured periodically on secondary MECC flasks.

Determination of LD_{50}. Two- to 5-day old sucklings, randomized on the day of injection, were inoculated ip with 0.05 ml aliquots of serial tenfold dilutions of MCMV. The animals were returned to nursing mothers in groups (litters) of nine, and at least two litters were inoculated with each dilution in each experiment. Mice were observed daily for 21

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days for signs of disease and the number surviving. The LD_{50} was calculated by the method of Reed and Meunch.

Immunization. Thirty- to 40-day old female mice were immunized using 3 ip injections given at weekly intervals, 2 of 4×10^4 PFU, and 1 of 4×10^5 PFU. Control mice were immunized on the same schedule and with an equal dilution of mouse salivary gland suspension prepared from uninoculated mice. These two groups of mice became the breeding stock for studies to determine the effects of immunization.

Plaque reduction neutralization assays. Sera from blood obtained from the retro-orbital venus plexus of adults or plasma collected from decapitated sucklings using heparinized capillary tubes were stored at 20°, and heat inactivated at 56° for 30 min prior to use. A volume of 0.3 ml of diluted serum or plasma was mixed with 0.3 ml of virus suspension containing 300–600 PFU MCMV. This mixture was incubated at 37° for 90 min, then 0.2 ml were inoculated into each of two Falcon flasks. Guinea pig complement, 10 units hemolytic activity in 10 μl was added to the reactants in some instances. After an absorption period of 2 hr at 37° the inoculum was removed and replaced with tragacanth overlay. The neutralization (plaque reduction) titer of a serum (or plasma) is the reciprocal of the highest dilution of that serum (or plasma) which reduced control plaque counts 50% or more.

Results. LD_{50}. The LD_{50} for suckling mice of the several stock pools of MCMV used in these experiments ranged from $10^{2.2}$ to $10^{3.2}$ PFU. A representative pool had $10^{7.6}$ PFU/ml, its LD_{50} for sucklings was $10^{2.8}$ PFU, and its LD^{50} for weanlings was 10^5 PFU. The LD^{50} for sucklings of the stock used in most of these experiments was 10^2 PFU, and that pool contained $10^{8.3}$ PFU/ml

CNS infection. The quantity of virus recovered from various organs after ip inoculation of a sublethal quantity of virus (average 36 PFU; range 2–75 PFU) is shown in Table I. Suckling mice were 1–4 days old when inoculated, and their organs were removed and tested at various times between 3 and 127 days thereafter. Virus was first detected in the CNS after it was recovered initially from the liver. Multiplication in the CNS peaked between 10 and 15 days and its extent was less than in other organs. Virus was recovered directly from 3 of 13 brains tested 60 and 80 days after inoculation, and from none of seven brains tested after 120 days. Using tissue explant culture techniques, virus was recovered only once from the brain when it was not detected by inoculation of MECC; CPE was observed in one flask seeded with brain tissue obtained at 127 days.

Histopathological examination of infected brains rarely revealed any changes. Once minimal perivascular cuffing and scattered nodules of glial proliferation with rare typical intranuclear inclusions were seen in a section of brain obtained 10 days after inoculation. Giant cells were not seen. Most brains were normal, including those obtained from mice 15 to 20 days after inoculation.

Effect of immunization on infection and mortality. In order to study the effect of immunization, four groups of suckling animals were tested. The first was born to and nursed by immunized females (I-I); the second was born to unimmunized females (either not injected at all or injected with normal salivary gland) and nursed by immunized females (NI-I); the third was born to immunized females and nursed by nonimmunized nursers

TABLE I. RECOVERY OF MCMV FROM SUCKLINGS AFTER INTRAPERITONEAL INOCULATION.[a]

Tissue	Days Post Inoculation							
	3	5	10	15	20	60	123	127
Brain	N[b]	1.4[c]	3.5	2.8	2.3	1.5	N	N[d]
Liver	2.7	3.6	4.5	3.9	2.6	N	2.6	N
Spleen	—[e]	4.1	4.7	4.0	2.7	3.9	N	N
Salivary gland	—	3.7	7.3	8.0	8.6	7.4	—	5.9

[a] Data are from eight experiments in which inoculum used averaged 36 PFU; the range was 2–75 PFU.
[b] Virus not detected in 10% w/v suspension.
[c] Average $\log_{10}$ PFU/g.
[d] See text (virus was recovered using explant method).
[e] Specimens not tested, or not obtained.

(I-NI); and the fourth group was born to and nursed by nonimmunized females (NI-NI). Litters were switched within 24 hr after birth, and sucklings were inoculated 3 days after switching. All sucklings were challenged ip with approximately 10^4 PFU (30 LD_{50}). The effect of immunization was assessed by determining the extent of virus multiplication in various organs and the mortality rates in the four groups of sucklings.

These results are provided in Tables II and III. In several experiments, each utilizing several pools of two or more organs, virus was not recovered from the brains or livers of animals in groups I-I or NI-I (Table II). Virus was recovered from but a single pool of spleens of animals in group NI-I which were sacrificed on day 10. Virus was recovered from 5 of the 12 pools of salivary glands obtained from mice of groups I-I and NI-I between days 10 and 20.

In striking contrast, virus was frequently recovered from the brains, livers, and spleens of that group of sucklings born to immune females but nursed by nonimmune, foster mothers (group I-NI). The quantity of virus was usually less than that recovered from sucklings born to and nursed by nonimmune females.

The reduction in mortality afforded by immunization is shown in Table III. Mortality in groups I-I, NI-I, and I-NI was 19, 16, and 26% respectively, in contrast to the 100% mortality induced in the NI-NI group; all were inoculated with 30 LD_{50}.

Serology. Plaque reducing antibody was detected in sera of females which were immunized, but not in those which had not been so treated. The median titer was 8.

Plasma obtained from three groups of I-I sucklings 19, 12–22, and 13 days after birth had titers of 8, 4, and <4 respectively. A pool of plasma obtained from suckling group NI-I 4 days after birth had no activity, whereas a pool obtained 14–19 days after birth had a titer of 4. The titer of another pool obtained 13 days after birth from that group (NI-I) was 4. Plasma from sucklings in groups NI-NI and I-NI had no plaque reducing activity. Complement had no discernable effect on the observed titers.

Discussion. The observation that CNS infection can follow ip inoculation of MCMV is not new. Selgrade and Osborn (7), and Kelsey *et al.* (8) have reported that CNS infections occur after ip inoculation of MCMV in both weanling and suckling mice. Selgrade and Osborn stated that no histopathological changes were seen in the brains of inoculated weanling C57BL mice. Our observations are similar in that very little was seen. The apparent paucity of white cells suggests that in these animals little inflammatory or immune response was present in the CNS at the time.

The immediate reason for documenting this CNS infection was to establish a baseline for ascertaining whether immunization of the mature female would confer protection from CNS infection to her offspring. Protection was conferred, and it is important that suck-

TABLE III. REDUCTION IN MORTALITY AFFORDED BY IMMUNIZATION.

Suckling group	No. dead / No. inoculated	Percent mortality
I-I	11/59	19
NI-I	22/137	16
I-NI	37/142	26
NI-NI	36/36	100

TABLE II. SUPPRESSION OF INFECTION AFFORDED BY IMMUNIZATION.[a]

Suckling group Mother–nurser	Organ tested and day postinoculation							
	Brain		Liver		Spleen		Salivary gland	
	Day 10	Day 14–20	Day 10	Day 14–20	Day 10	Day 14–20	Day 10	Day 14–20
I-I	0/6[b]	0/6	0/3	0/4	1/3 (2.0)[c]	0/4	1/2 (3.6)	2/3 (4.1)
NI-I	0/7	0/5	0/4	0/4	0/4	0/3	0/3	2/4 (4.4)
I-NI	4/7 (3.0)	2/2 (2.8)	3/4 (3.6)	3/4 (3.6)	4/4 (4.8)	2/2 (4.4)	3/4 (7.8)	3/3 (8.2)
NI-NI	Day 6[d] 1/1 (3.0)		Day 6 1/1 (5.9)		Day 6 1/1 (6.3)		Day 6 1/1 (4.6)	

[a] Challenge inoculum = 30 LD_{50}.
[b] Number pools positive/Number pools tested.
[c] Average titer (log PFU/g) of positive pools.
[d] All mice in group NI-NI died by end of 6th day.

lings born of females which were not immune, but nursed by immune foster mothers were afforded as much protection as those born to and nursed by immune females. Moreover, they received more protection than those sucklings which were born to immune mothers but nursed by nonimmune foster mothers. Organs of sucklings born to mothers which had been immunized but nursed by those who had not been, generally contained less virus, and their mortality was less than that of controls. They were, nevertheless, much less protected than suckling mice which had been nursed by immune mothers. Mortality has been directly associated with the extent of viral multiplication in the viscera in MCMV (9). The presence of a reduction in mortality in animals in which antibody was not detected is interesting, but not surprising. Our methods may be unable to detect significant quantities of protective humoral antibody, or it may be that plaque reducing antibody is not a significant protective factor (10).

It does seem reasonable to conclude that the suckling obtained an essential protective factor (or factors) from an immune foster mother. This observation is like that reported by many authors for many infections in mice as reviewed by Brambell (5). It is known that immune globulins are secreted in murine breast milk and are absorbed by the suckling mouse. Fahey and Barth found that IgG was present in low but detectable amounts in newborn mice, and in colostrum (11). The levels in the suckling rose rapidly during the first week then fell rapidly between the 2nd and 4th weeks of life. By 8 weeks, the level had risen to that of the adult mouse. Our results are consistent with those observations in that the sera of immune mothers contained plaque reducing activity as did the plasma of sucklings in the protected group(s) when it was obtained after a period of nursing.

Other factors might be operating. There is evidence in mice that maternal cells can cross the placenta (12), and that other factors may induce immune alterations (13). Moreover, cells in breast milk capable of mediating cellular immunity can cross the neonatal gut in rats (14). Our crude attempts to obtain sufficient quantities of milk to study cellular and humoral factors were unsuccessful, and it should be noted that we did not determine whether MCMV was being excreted in breast milk.

There is some knowledge of the nature of immunity to MCMV. Selgrade and Osborn have shown that CBA/J mice were more resistent to MCMV than C57BL6J mice, that suckling C57BL6J mice were more susceptible than adults, and that the susceptibility of the suckling was reduced by experimental transfer of syngeneic macrophages or nonimmune lymphocytes from adult mice (7). There was no difference in the *in vitro* response to MCMV of macrophages from these various sources, thus this was different than what Johnson had shown for herpes virus (15). Although differences in susceptibility to MCMV (as measured by comparison of LD_{50}s) were shown to exist between animals that did not apparently differ in terms of their B and T cell function, subsequent studies by Selgrade *et al.* indicated that both T and B cells have some role in host defense against MCMV (16).

Booss and Wheelock have shown that acute MCMV infection impairs T-cell function (17) and that MCMV itself may be the significant suppressive factor (18). Starr and Allison have shown that MCMV induced death of congenitally athymic nude mice is prevented by T-lymphocytes (19).

Protection from human CMV infection and disease seems as complex as MCMV infection. Cytomegalic inclusion disease rarely, if ever, occurs in infants born after an affected baby (20). Virus excretion does occur in mothers who have complement fixing and neutralizing antibody, and their babies can become infected *in utero* with virus susceptible to those antibodies (10). Cell mediated immunity as measured by cytotoxicity or lymphocyte proliferation is suppressed in HCMV infection, but these findings have not been made in situations which could help explain what factors are essential for protection of unborn or neonatal infants.

The purpose of this discussion is not to provide a detailed analysis from which one might conclude which immune response is the more likely to be operative, but rather to review the possible factors which will have to be evaluated in order to learn how protection was conferred.

The experimental model described provides an opportunity to study the immune

response and protection to MCMV in a context analogous to the human situation which results in mental retardation.

Summary. Female weanling mice were immunized with suspensions of MCMV infected salivary glands, then mated. Protection was conferred to their young by breast feeding, and to a lesser extent it was acquired *in utero.* Protection was manifested by a reduction in mortality, and by a reduction in the quantity of virus recovered from brain, liver, spleen, and salivary gland after challenge with 30 LD_{50}. Neutralization antibody (plaque reducing activity) was present in serum of immunized females and in the plasma of sucklings born of and nursed by them, or those only nursed by them. It was not present in unimmunized females or in sucklings born of but not nursed by immunized females.

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Influence of Feeding Frequency on Body Weight and Glucose Tolerance in the Pig[1] (40090)

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An alteration in the meal pattern of animals has been reported to influence body composition, glucose tolerance and circulating lipid levels in a number of studies (1–4); however, these observations have not been confirmed in other studies (5–8). The present study was designed to evaluate the influence of meal pattern on body weight, body composition, glucose tolerance and several plasma metabolites in growing pigs. The pig was chosen as the animal model because of the physiological similarities between the pig and the human (9).

Materials and methods. Four castrated male Yorkshire pigs and four female Yorkshire–Hampshire crossbred pigs were assigned to each of two treatments. Pigs were housed in individual stalls (46 × 137 cm) with concrete-slat floors. Water was available ad libitum from an automatic watering device.

Eight pigs were allowed access to diet for 4 hr daily for the first 7 days and then were fed one 4-hr meal every 48 hr for the next 165 days. Based on the average food intake of the four castrated male pigs, each of the four other castrated male pigs were fed an identical total amount of food but as two equally-sized meals per day. Likewise, four female pigs were similarly pair-fed twice daily. Pigs fed two meals per day consumed their entire meal within a few minutes after it was offered. Pigs were weighed every 8 days. The corn–soybean meal based diet contained 16% protein for the first 120 days of the experiment and 13% protein thereafter. The diet was initially fed in ground form but was pelleted after the first 60 days to facilitate diet weigh-back. One pig fed four meals per 48 hr died; however, the cause of death was not related to the experiment.

An intravenous glucose tolerance test was conducted after the pigs had been meal-fed for 150 days. Each pig received 900g diet 24 hr prior to the glucose tolerance test. Pigs were restrained in a padded sling prior to the test. An indwelling needle catheter[4] was established in an ear vein and 0.75 g glucose (40% glucose in saline) per kg body weight was injected. Blood samples were obtained from the needle catheter at timed intervals after the glucose load.

To obtain estimates of the levels of several plasma metabolites blood samples were obtained by puncture of the anterior vena cava just prior to a regularly scheduled meal. Plasma glucose,[5] triglycerides[6] and cholesterol (10) levels were determined as described.

Biopsy adipose tissue samples were obtained (11) 4 hr after a regularly scheduled meal. Malic enzyme (EC 1.1.1.40) activity was determined on the 50,000*g* supernatant fluid (12). Protein content of the supernatant fluid was determined by the method of Lowry *et al.* (13).

Pigs were killed and carcass data determined by standard procedures (14). The weight of muscle was also calculated (15).

The data were treated statistically by the student's *t* test.

Results and discussion. Within 20 days the pigs were each able to consume 1 kg of diet in a 4-hr period (Fig. 1). Food intake increased to 4 kg in a 4-hr period by the end of the study. Pigs fed more frequently consumed their meals within a few minutes after it was offered. Body weight of the pigs remained relatively constant for the first 30–40 days and then increased (Fig. 1). Total body

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[4] Becton, Dickinson & Co., Rutherford, New Jersey.

[5] Glucostat, Worthington Biochemical Corp., Freehold, New Jersey.

[6] Technicon Autoanalyzer Triglyceride Procedure, Tarrytown, New York.

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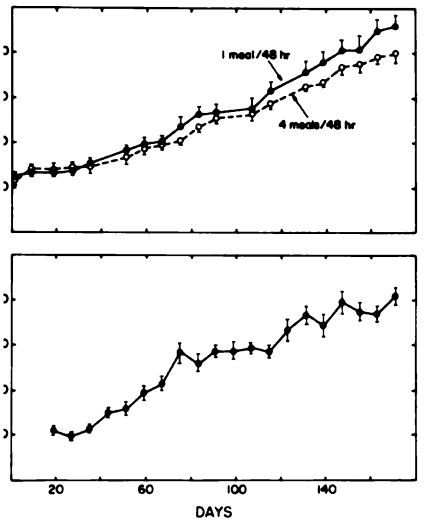

1. Body weight and food intake of pigs. Values
t mean ± SEM for eight pigs fed one meal and
gs fed four meals per 48 hr. Pigs fed four meals
r were pair-fed to those receiving one meal per

gain was not influenced by the feeding
ıcy (Table I). The pigs gained approx-
r 0.35 kg per day. They would have
ıpected to gain approximately 0.65 kg
y had they been fed *ad libitum*. Pigs
lso been pair-fed one versus several
per day; no differences in carcass
gain were observed (16–18). Likewise,
d one or four meals per 48 hr exhibited
erences in body weight (8). These ex-
nts as well as others (2, 7) suggest that
ration in meal frequency does not in-
: body weiglit, provided the animals
r-fed.

nates of body fat and muscle content
pigs were obtained at the end of the
Table I). Meal frequency did not influ-
.e results. Rats force-fed two meals per
:umulate more body fat than *ad libitum*
ı (1, 2). It has also been suggested that
s consuming fewer meals per day have
:ncy to be fatter (1). However, in the
: study with pair-fed pigs and in other
studies with dogs (8) and rats (7) meal
ıcy did not influence body fat. The
's) for the pronounced influence of
·equency on body fat when rats are
:d, as opposed to minimal influences
ınimals are pair-fed meals, remains to
idated.

were fed a 900 g meal 24 hr prior to

TABLE I. INFLUENCE OF MEAL FREQUENCY ON BODY WEIGHT GAIN AND ON CARCASS MEASUREMENTS IN THE PIG.[a]

	Meals per 48 hr	
	1	4
Initial body wt, kg	24.14 ± 1.79	23.63 ± 1.64[b]
Body wt gain, kg	63.47 ± 4.29	54.22 ± 2.84[b]
Carcass wt, kg	60.40 ± 4.03	55.29 ± 3.08[b]
Backfat thickness, cm	1.98 ± 0.26	1.71 ± 0.40[b]
Fat trim, kg	5.75 ± 0.84	4.49 ± 0.74[b]
Longissimus dorsi area, cm^2	30.2 ± 0.80	26.6 ± 1.40[b]
Muscle, kg	36.60 ± 1.44	32.23 ± 2.35[b]

[a] Mean ± SEM for eight pigs fed one meal and seven pigs fed four meals per 48 hr.

[b] Values were not significantly different from pigs fed one meal per 48 hr, $P > 0.05$.

the glucose tolerance test to standardize the postabsorptive state at the time of the glucose load. All pigs cleared the glucose with equal efficiency; meal frequency did not influence the changes in plasma glucose following the glucose load (Fig. 2). Similar results have been reported in meal-fed dogs (8). Likewise, an alteration in meal pattern had only a minimal influence on glucose tolerance in the chicken (6) and rat (5) when food intake prior to the test was controlled. Failure to control food intake among the treatment groups may alter the results (5, 6, 8). Humans have been reported to exhibit an impaired glucose tolerance when meal frequency is decreased (1, 4). These results have not been reproduced in the rat, chicken, dog or pig. It is not possible at this time to determine the cause for these differences in response to meal frequency.

Plasma glucose, triglyceride and cholesterol levels were determined just prior to a regularly scheduled meal (Table II). Thus, 44 hr had elapsed since pigs fed once every other day had finished a meal whereas pigs fed more frequently finished a meal 12 hr prior to the sampling. The plasma metabolites measured were not influenced by meal frequency. In a previous report serum cholesterol levels tended to be slightly higher in pigs fed ad libitum than in pigs fed one 2-hr meal per day (19). Similarily, dogs fed two meals per day had higher plasma cholesterol levels than did dogs fed one meal every other day (8). However, in a number of other studies (1, 4, 17) circulating cholesterol levels are either not changed or are decreased as meal frequency is increased. It appears that meal-frequency generally has a minimal influence

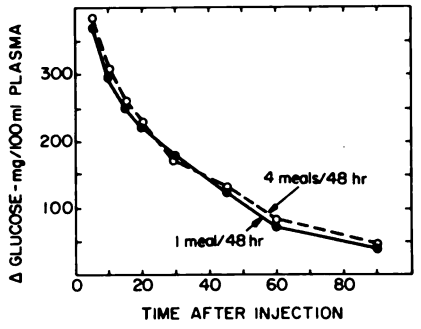

FIG. 2. Intravenous glucose tolerance in pigs fed one or four meals per 48 hr. All pigs received 900 g diet 24 hr prior to the test. Each pig was injected with 0.75 g glucose (a 40% glucose solution in saline) per kg body weight. Blood samples were obtained at timed intervals during the next 90 min. Results are expressed as increase in plasma glucose above zero-time values. Each point represents the mean for eight pigs fed one meal and seven pigs fed four meals per 48 hr. Zero-time values were 88 ± 7 and 71 ± 2 mg glucose per 100 ml plasma for pigs fed one and four meals per 48 hr, respectively.

TABLE II. INFLUENCE OF MEAL FREQUENCY ON SEVERAL PLASMA METABOLITES AND ON MALIC ENZYME ACTIVITY IN PIG ADIPOSE TISSUE.[a]

	Meals per 48 hr	
	1	4
Plasma.[b]		
Glucose	99 ± 3	102 ± 5
Triglycerides	48 ± 3	59 ± 8
Cholesterol	108 ± 6	98 ± 4
Adipose.[c]		
Malic enzyme	118 ± 12	66 ± 6[d]

[a] Mean ± SEM for eight pigs fed one meal and seven pigs fed four meals per 48 hr.

[b] Values expressed as mg per 100 ml plasma.

[c] Enzyme activity expressed as nmoles substrate utilized per minute per mg protein at 25°.

[d] Significantly different from values obtained when one meal per 48 hr was fed, $P < 0.05$.

on circulating cholesterol levels. Plasma triglyceride levels were not altered in the pigs in agreement with other reports (4, 8).

Malic enzyme activity in the pig adipose tissue was assayed as an indicator of lipogenic capacity. Pigs fed less frequently exhibited an elevated enzyme activity (Table II). The magnitude of the difference in enzyme activity between the two groups is similar to a previous report (14). The elevated lipogenic capacity of the pigs fed less frequently would allow a portion of the energy in the high-carbohydrate diet to be stored as fat immediately after the meal. This fat could then be mobilized to supply a source of energy during the postabsorptive state; consequently, the increased lipogenic capacity would not necessarily result in increased body fat.

Summary. Pigs were pair-fed one or four meals per 48 hr for 5.5 months. Meal frequency did not influence body weight gain, body composition, glucose tolerance or plasma glucose, cholesterol or triglyceride levels. Pigs fed less frequently exhibited elevated malic enzyme activity in adipose tissue.

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mic Changes during Acute Salicylate Intoxication in Conscious Dogs (40091)

IN C. WANG, NANCY J. MUETING, AND KENNETH L. GOETZ

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ı salicylate intoxication continues
nmon clinical problem, especially
s, relatively few studies have been
on the cardiovascular effects of
A report of Tenney and Miller (1)
the most cited reference that deals
rculatory effects of salicylates, yet
was performed before beta adre-
king agents were available. Ten-
iller (1) concluded that salicylates
ect stimulating action on cardiac
ch probably is independent of au-
rvous activity. For the most part
ascular effects of salicylates have
d in the anesthetized dog (1, 2);
owing evidence, however, that the
log responds to certain physiolog-
armacological stimuli in a manner
ent from that of the anesthetized
or the above reasons, we have
in detail the cardiovascular and
esponses that are elicited by the
s infusion of large doses of sodium
nto the conscious dog.

Experiments were performed on
nongrel dogs weighing an average
Each dog was prepared for the
s during a preliminary operation
thesia with sodium pentobarbital
body weight with supplements as
The chest was entered aseptically
side, and a flow probe was placed
ascending aorta. Vinyl catheters
d in the descending aorta, pulmo-
, and superior vena cava by the
of Herd and Barger (4). In addi-
theter was inserted into the left
ough a small tributary of a pul-
in. The proximal ends of the cath-
low probe cable emerged from the
nt to the spinal column and were
in a styrofoam block. The wound
with sutures and sealed with a
sing. During the operation each
eived 500 ml of 5% dextrose in water iv; this solution also contained one million units of aqueous penicillin G. Postoperatively, the animals received 300,000 U of procaine penicillin G im daily for 5 days. All catheters were flushed with saline three times each week and filled with a solution of sodium heparin in saline (1000 U/ml). A nylon jacket protected the catheters and flow probe.

Each dog was allowed to recover from the operation for at least 1 week and was trained to rest quietly in an animal stand. During each experiment pressures in the aorta, pulmonary artery, left atrium, and superior vena cava were monitored continuously using Statham P23Db transducers. Cardiac output was measured with an electromagnetic flowmeter and a circuit that automatically zeroed the baseline of aortic flow on a beat-to-beat basis (5). All pressures and flow signals were recorded on an oscillographic recorder. Data were analyzed by an automatic data averager that accurately computed the time-integrated mean value of up to seven inputs and printed a copy of the results (6). Total peripheral vascular resistance and pulmonary vascular resistance were calculated by standard methods (7).

Sodium salicylate was dissolved in distilled water to produce an isotonic solution (24 mg/ml) which was infused intravenously at a rate of 20 mg/kg/min. Three different doses of sodium salicylate were infused into each dog according to the following sequence: day 1, 100 mg/kg; day 2, recovery period; day 3, 200 mg/kg; day 4 and day 5, recovery period; day 6, 200 mg/kg after pretreatment with propranolol (2 mg/kg); day 7 and day 8, recovery period; day 9, 300 mg/kg. In addition, isotonic saline in a volume comparable to each of the three salicylate doses was given to some animals at the same rate to examine the effect of the volume load itself on the cardiovascular system. The recovery periods were adequate to allow dissipation of the

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effects of the previous dose of salicylate as evidenced by a normal acid-base status and undetectable levels of plasma salicylate in all control periods.

Rectal temperature of the animal was continuously monitored with a thermistor probe. Arterial blood samples were drawn anaerobically. Plasma pH, PO_2, and PCO_2 were measured with appropriate electrodes maintained at 37°. Appropriate coefficients were used for temperature correction for pH (8), PO_2, and PCO_2 (9). Plasma bicarbonate values were calculated from pH and PCO_2 using the Henderson–Hasselbalch equation. Plasma salicylate concentration was determined according to the method of Trinder (10).

The data in the table and figures are expressed as the mean ± SE. The paired *t* test was used to determine whether the changes induced by salicylate infusion were significantly different from control values. The analysis of variance (*F* test) and a sequential variant of the Q method (11), originally designed by Newman and Keuls, were used to determine whether the peak changes induced by a given dose of salicylate differed significantly from the response at other dose levels. All *P* values of greater than 0.05 were considered to be statistically insignificant.

Results. The mean hemodynamic responses of 11 conscious dogs to the intravenous infusion of sodium salicylate (200 mg/kg) are summarized in Fig. 1. Heart rate, cardiac output, and pulmonary arterial pressure increased following salicylate infusion while total peripheral resistance decreased. The maximum changes in these variables occurred at the end of the 10-min infusion period, but the values remained significantly different from their respective control measurements throughout the entire experiment. The increase in cardiac output was caused almost entirely by an increase in heart rate; stroke volume remained essentially unchanged with the exception of a slight increase that occurred 5 min after the infusion began. Aortic pressure increased initially and then gradually decreased to below control measurements approximately 1 hour after the onset of the infusion. Both central venous pressure and left atrial pressure gradually decreased with time. Pulmonary vascular resistance was not altered significantly by salic-

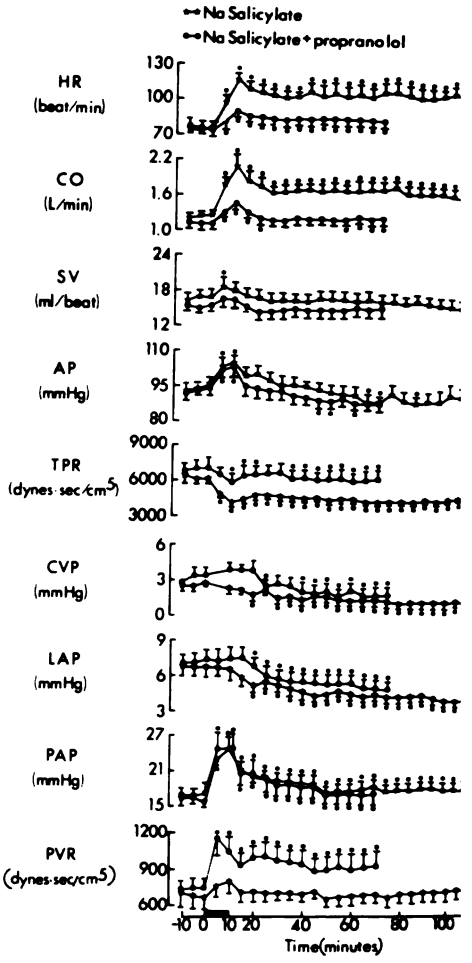
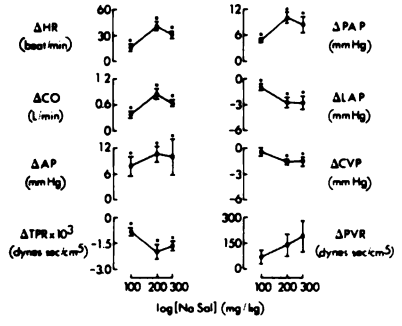


FIG. 1. Time course of the mean cardiovascul sponses following the intravenous infusion of so salicylate (200 mg/kg) with and without pretrea with propranolol (2 mg/kg). The 10-min infusion p is denoted by the bold horizontal line. Abbrevi used are: HR, heart rate; CO, cardiac output; SV, s volume; AP, aortic pressure; TPR, total peripher sistance; CVP, central venous pressure; PAP, pulm arterial pressure; LAP, left atrial pressure; and pulmonary vascular resistance. An asterisk indicate ues that are significantly different from control me ments ($P < 0.05$ by paired *t* test, $n = 11$).

ylate infusion. Infusion of a volume of s equal to that of the salicylate infusion f to produce any significant cardiovas changes in the five dogs tested (not sho thus indicating that the changes obse after salicylate infusion were caused b substance itself, and not by the volum fluid added. Figure 1 also illustrates the e of sodium salicylate after beta adren

ade (propranolol, 2 mg/kg). The maxi- increases in heart rate and cardiac out- licited by salicylate were attenuated by)ximately 60% after pretreatment with anolol, whereas the decrease in total heral resistance was attenuated by ap- mately 35%. Pretreatment with propran- ılso altered the response of the pulmo- vasculature to the salicylate infusion as nced by the significant increase in pul- ıry vascular resistance induced by salic- after propranolol was given. Other car- scular responses elicited by salicylate relatively unaffected by pretreatment propranolol.

e peak hemodynamic changes elicited ree doses of sodium salicylate are shown g. 2. Salicylate produced significant in- es in heart rate, cardiac output, aortic ure, and pulmonary arterial pressure, ignificant decreases in total peripheral ance, left atrial pressure, and central ıs pressure. Pulmonary vascular resist- increased slightly after each of the three of salicylate, but the increases did not significantly from control values ($P >$ Although all cardiovascular changes ared to have a dose-dependent tendency, ıalysis of variance indicated that only variables, ΔHR, ΔCO, ΔTPR, and ΔPAP ed significant dose-dependent changes 0.05). The maximal response of these variables to a dose of 200 mg/kg was icantly greater than the response to 100 mg/kg ($P < 0.05$). On the other hand, the responses to a dose of 300 mg/kg were slightly less than the responses to a dose of 200 mg/kg, but the differences did not reach statistical significance ($P > 0.05$).



2. Peak cardiovascular changes produced by the enous infusion of three doses of sodium salicylate. erisk indicates values that are significantly differ- om their respective control measurements ($P <$ y paired t test). The number of animals studied), 11, and 9 for doses of 100, 200, and 300 mg/kg, tively. Abbreviations used are the same as those in

Data of plasma salicylate concentration, body temperature, and arterial plasma pH, PCO_2, $\{HCO_3^-\}$, and PO_2 are shown in Table I. The concentrations of salicylate in plasma at 15 min after infusion were 24 ± 1, 42 ± 1, and 60 ± 2 mg/100 ml of plasma for doses of 100, 200, and 300 mg/kg, respectively. Plasma salicylate concentration then decreased with time as shown in Table I. The mean control body temperature of the dog ranged from 39.3 to 39.6°; it was not significantly altered by any of the three doses of salicylate. Arterial plasma pH and PO_2 increased following salicylate administration while PCO_2 decreased and $\{HCO_3^-\}$ decreased slightly or did not change. We regularly observed an increase in the respiratory rate after salicylate was given. Pretreatment with propranolol did not alter the acid-base and PO_2 changes elicited by salicylate (not shown in Table I). The emetic threshold of our conscious dog, at a comparable infusion rate, was about equal to that reported in the conscious cat (12); vomiting occurred in all dogs during the 10-min infusion of salicylate at doses of 200 mg/kg or higher.

Discussion. In conscious dogs, sodium salicylate produced an increase in cardiac output, a decrease in total peripheral resistance, and a biphasic response in aortic pressure (an initial increase followed by a decrease to below control values). These hemodynamic changes are generally comparable to those reported in the anesthetized dogs (1). They differ, however, in that the cardiac output of our conscious dogs was increased by an increase in heart rate whereas this was accomplished by an increase in stroke volume in the anesthetized dogs (1). In conscious dogs, sodium salicylate also produced an increase in pulmonary arterial pressure and decreases in left atrial pressure and central venous pressure. The increase in pulmonary arterial pressure was largely attributable to an increase in pulmonary blood flow under normal conditions. The decreases in left atrial pressure and central venous pressure were probably due to vasodilation of the peripheral vasculature. These hemodynamic changes elicited by sa-

TABLE I. PLASMA SALICYLATE CONCENTRATION, BODY TEMPERATURE, AND ARTERIAL pH AND BLOOD GASES AFTER iv Na SALICYLATE.

	Dose (mg/kg)	n	Control	60 min	120 min
{Sal} pl[a] (mg%)	100	13	0	21 ± 1	19 ± 1
	200	12	0	39 ± 1	36 ± 1
	300	9	0	55 ± 3	50 ± 2
Body temp. (°C)	100	12	39.4 ± 0.2	39.5 ± 0.1	39.2 ± 0.2
	200	11	39.5 ± 0.3	39.5 ± 0.2	39.3 ± 0.2
	300	6	39.3 ± 0.3	39.3 ± 0.2	38.9 ± 0.1
pH	100	12	7.42 ± 0.01	7.45 ± 0.01*	7.45 ± 0.01
	200	10	7.42 ± 0.01	7.45 ± 0.01*	7.48 ± 0.01*
	300	6	7.40 ± 0.01	7.49 ± 0.02*	7.49 ± 0.02*
PCO_2 (mmHg)	100	12	35.2 ± 1.1	31.2 ± 0.8*	31.1 ± 0.7
	200	11	36.1 ± 1.1	32.1 ± 0.7*	29.8 ± 0.8*
	300	6	35.0 ± 0.2	28.3 ± 1.1*	28.3 ± 0.7*
$\{HCO_3^-\}$ (m*M*)	100	12	22.8 ± 0.7	21.2 ± 0.5*	20.7 ± 0.5
	200	10	22.4 ± 0.5	21.8 ± 0.4	21.2 ± 0.9
	300	6	21.9 ± 0.5	21.4 ± 0.7	21.1 ± 1.0
PO_2 (mmHg)	100	12	90.2 ± 1.7	93.6 ± 1.3*	95.0 ± 2.3
	200	11	91.9 ± 1.7	95.9 ± 1.2*	96.3 ± 1.2*
	300	6	88.1 ± 2.3	96.5 ± 1.8*	92.5 ± 3.5

[a] Plasma salicylate concentration.
* Significantly different from control ($P < 0.05$).

licylate were dose-dependent and a maximum response was obtained at a dose of 200 mg/kg.

The mechanism of the hemodynamic changes elicited by salicylate is not entirely clear. Tenney and Miller (1) proposed that the increase in cardiac output elicited by salicylate is independent of any nervous influence and is mediated through a direct stimulation of the salicylate ion on the myocardium. Their conclusion was based upon data indicating that the alpha adrenergic antagonist, phenoxybenzamine, and the ganglionic blocker, tetraethyl ammonium chloride, did not prevent the initial pressure increase elicited by salicylate. Supporting evidence was provided by others who found that intravenous infusion of salicylate caused a typical positive inotropic response after procaine had been infused epidurally to produce sympathetic block (2). In addition, low concentrations of salicylate have been reported to increase contractility of the isolated heart (13). These results along with our data indicating that propranolol did not completely abolish the salicylate-induced increases in heart rate and cardiac output support the hypothesis that salicylate does exert a positive inotropic effect directly on the myocardium. On the other hand, since beta adrenergic blockade with propranolol significantly attenuated the increases in heart rate and cardiac output caused by salicylate, our results provide some evidence that these responses may be attributed partly to the activation of beta adrenergic receptors. This view is consistent with the observation that large doses of salicylate activate central sympathetic centers (13). Our results also suggest that salicylate may activate the beta adrenergic receptors in the peripheral vasculature because pretreatment with propranolol attenuated the decrease in total peripheral resistance elicited by salicylate. Moreover, the increase in pulmonary vascular resistance induced by salicylate after pretreatment with propranolol may imply that salicylate normally activates both vasodilator (beta) and vasoconstrictor mechanisms. That is, the beta receptor blockade may have eliminated the vasodilation and thus unmasked the vasoconstriction. It should be kept in mind, however, that propranolol may exert its effect through nonspecific membrane actions or through effects on the central nervous system. Therefore the question regarding the effect of salicylate on the activation of beta adrenergic receptors is not completely settled.

Sodium salicylate did not alter the body temperature of our conscious dogs even though comparable doses of salicylate produce hyperthermia in the anesthetized dog

alicylate increases heat production by
)ling oxidative phosphorylation in mi-
dria (15) with a consequent increase in
ı consumption (14), but it also increases
issipation through peripheral vasodi-
The net effect on body temperature
epends on the balance of these two
ng changes, and it appears that this
e is altered by anesthesia.
:ylate produces respiratory alkalosis in
nscious dogs as evidenced by an in-
in arterial plasma pH, a decrease in
and no significant change in bicarbon-
ıcentration. Thus the respiratory re-
of these animals is similar to that of
l human subjects given comparable
)f salicylate (16). Since CO_2 production
:ased following salicylate administra-
, 14), the observed decrease in arterial
suggests that the salicylate infusion
:ed alveolar ventilation. Salicylate also
:es hypocapnea in anesthetized dogs,
mild bicarbonate deficit is superim-
on the respiratory alkalosis (14). The
e of the bicarbonate deficit in our ex-
:nts may be attributable to the vomiting
occurred consistently when doses of
g/kg or higher were given.

mary. The hemodynamic changes fol-
intravenous infusions of sodium salic-
/ere measured in conscious dogs. The
ı salicylate produced dose-dependent
:es in heart rate, cardiac output, and
nary arterial pressure, and a dose-de-
ıt decrease in total peripheral resist-
vith maximum responses being ob-
at the dose of 200 mg/kg. Sodium
ıte also produced decreases in left
pressure and central venous pressure
biphasic response in aortic pressure.
ıtment with propranolol significantly attenuated the increases in heart rate and cardiac output, as well as the decrease in total peripheral resistance elicited by salicylate, thus implying that the salicylate-induced cardiovascular responses are mediated partly through the activation of beta adrenergic receptors.

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Control of A–V Shunt and Capillary Circuits in the Dog Hindpaw (40092)

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The distribution of blood flow in peripheral vascular areas is known to be influenced by neural (1–4), myogenic (5) and hormonal (6, 7) mechanisms. The dog hindpaw is unique for studying these controls because there are two major vascular circuits present connected in parallel. These are the nutritional or capillary circuits and the nonnutritional or arterio-venous anastomosis circuits. The dog hindpaw vasculature receives its innervation from three separate nerves that affect the arteries, small vessels and veins in distinctly different combinations (8, 9). The deep fibular nerve and the tibial nerve are reported to constrict arterial and small vessel segments when stimulated. Superficial fibular nerve stimulation causes small vessel segment and venous segment constriction.

The present study is to determine the effects of stimulation of each of these nerves at several frequencies on blood flow and volume distribution under conditions of controlled perfusion pressure.

Methods. Seventeen mongrel dogs averaging 18 kg in weight were pretreated with 10 mg/kg morphine sulfate and anesthetised with 15–20 mg/kg pentobarbital. The right hind paw was neurally and vascularly isolated at the ankle joint. The cranial tibial artery, saphenous vein, the tibial nerve, superficial fibular nerve and deep fibular nerve were isolated. The nerves were doubly ligated and sectioned. The skin flap produced by the isolation procedures was used as a seal for an air-filled volume recorder (plethysmograph).

The animals were given 10 mg/kg heparin. The saphenous vein was cannulated so that the blood flow passed through a scintillation detector. Venous outflow pressure was altered by a Starling Resistor for CFC measurements. The cranial tibial artery was perfused under controlled pressure conditions from a femoral artery by means of tubing having sidearms for measurement of perfusion pressure and injection of indicators. The perfused blood was kept at 37° by means of a heat exchanger. During the cannulation procedure, blood flow to the hindpaw was interrupted for less than one minute.

Stimulations of the superficial fibular nerve (60 volts, 0.3 msec duration), the deep fibular nerve (40 V, 0.3 msec duration), and the tibial nerve (60 V, 0.3 msec duration) were made at 0.1, 1, 5, and 11 Hz. Stimulation frequencies higher than 1.1 Hz reduced blood flow to levels where the indicator data were not reproducible. Elapsed time of stimulation of each nerve averaged nine minutes. Only experiments in which the blood flow change was maintained for the entire stimulation period are included.

The duration of the experiments was variable. Sufficient postsurgical time was allowed for the animals to stabilize and the hindpaws to become isovolumetric. It was unnecessary to immobilize the animals with a curarelike drug since stimulation of the nerves did not consistently elicit movement of the paw and in those instances where some movement did occur, the data was not different from paws that did not show contractions.

All measurements were made during control periods and during each period of nerve stimulation and recorded on an Electronics for Medicine DR-8 recorder. Systemic arterial pressure, paw perfusion pressure and venous outflow pressure were monitored using Statham P23AA transducers. Total paw volume changes were measured with the air-filled plethysmograph connected to a Statham P23-2D-300 pressure transducer. The sensitivity of the system was 2 cm of recorder deflection per milliliter change in paw volume. Arterial inflow was monitored with a Carolina Medical electromagnetic flowmeter. Venous outflow was measured by a timed collection of the effluent in a graduated cylinder (a separate collection was made for each injected isotope). Blood containing isotope was not returned to the dog but rather

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uous infusion of homologous, hepa-
donor blood was made at approxi-
he same rate as venous outflow. The
of donor blood infused was sufficient
tain the dog's mean arterial pressure
t and averaged 1500 ml per experi-
apillary filtration coefficient (CFC)
asured by rapidly increasing the ve-
itflow pressure 10 mmHg with the
resistor. This produces an initial
crease in paw volume due to venous
on followed by a slower increase due
ard filtration of fluid (5, 10). The
g equation using this filtration slope
e plethysmograph record and the ve-
ssure change was used. The assump-
s made that 80% of the venous pres-
rement is transmitted to the capillar-

100 (filtration rate)/0.8

enous pressure change)(paw weight)

onceivable that the 0.8 factor would
accurate under all conditions. Since
compliance and radius may vary with
ental conditions the fraction trans-
would be expected to be greater with
ilation and less with venous constric-

e vascular volumes were measured
d cells-^{51}Cr and albumin-^{131}I as indi-
The red cells were labeled as previ-
ported. A 0.25 ml aliquot of these
-5 mCi ^{51}Cr) was injected as a bolus
dearm of the arterial cannula as was
of albumin-^{131}I (0.3–0.5 mCi ^{131}I).
licators were injected separately and
ultaneously. The time-concentration
f the venous effluent were monitored
viously described hole-through scin-
detector and rate meter (10). Each
n was made in less than 1 sec and
h this transiently changed pressure
v the recording showed no persistent
in vascular resistance. Completion of
tion curve was ascertained by return
ecording to background levels. Indi-
covery was subsequently measured by
ning the radioactivity of a mixed sam-
e venous effluent collected during the
f injection. Blood flow and vascular
were calculated from the time-concentration curves by the method of Hamilton *et al.* (11). There was no distortion of the curves due to recirculation since all the indicators were collected in graduated cylinders and not returned to the animals. The volume of the system from the point of injection to the point of detection by the probe was calculated by multiplying the mean transit time (MTT) in seconds by the measured flow in milliliters per second. The vascular volume was then calculated by subtracting the volume of the inflow and outflow tubing from the total volume. The changes in vascular volume were compared to each other as well as to those noted from the volume recorder.

Capillary diffusion capacity (PS) was calculated from the extraction of 0.25 ml bolus injection containing 3–5 mCi of ^{86}RbCl using the following equation: $PS = -Q \ln (1-E)$ in which Q is the blood flow in milliliters per minute per 100 g tissue and E is the extraction of ^{86}RbCl. The extraction was measured by collecting the venous outflow in a graduated cylinder and subtracting the amount of indicator collected from the amount injected. Our method differs from that of Renkin (12) in that a bolus injection was used rather than a constant arterial infusion. Theoretically, E should be the same for either method if uptake by the cells can keep pace with transcapillary diffusion. If the tissue cell membranes limit interstitial clearance of the indicator, back diffusion would introduce an error when flow is changed from control. Comparison of the time-concentration curves of ^{86}Rb and albumin-^{131}I by the method of Martin and Yudilevich (13) indicate that back diffusion was minimal in these types of experiments.

Even though it has been reported by Renkin that PS may not adequately reflect changes in capillary surface area when flow is changing, the calculation was made under constant pressure perfusion. This was done to determine how PS changes compare with CFC changes which are not considered to be as blood flow dependent.

All data are reported with standard error of the means (SEM). Appropriately related points were evaluated with a paired-*t* test.

Results. Hemodynamic changes (Fig. 1). The hindpaws perfused under conditions of controlled pressure (93 ± 4 mmHg) exhibited progressive decreases in blood flow and in-

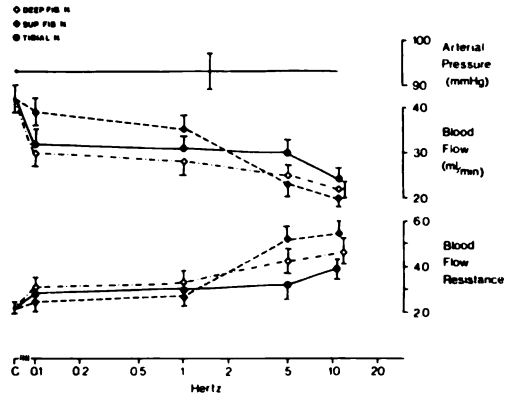


FIG. 1. Hemodynamic responses to several stimulation rates of the superficial fibular nerve, deep fibular nerve and tibial nerve during controlled pressure perfusion of 17 experiments. Brackets indicate ±SEM.

creases in resistance with stimulation frequency. The responses of all three nerves were similar.

Vascular and tissue volume changes (Fig. 2). Stimulation of the superficial fibular nerve at 0.1 Hz caused a small but significant ($P < 0.05$) decrease in total tissue volume but no change in vascular volume as measured by red cells-^{51}Cr and albumin-^{131}I. Total tissue volume increased at 1 Hz and 5 Hz ($P < 0.01$) and remained at that level at 11 Hz. Edema was easily observed at the higher frequencies. Vascular volume decreased minimally but insignificantly with increasing rates of stimulation.

Stimulation of the deep fibular nerve and the tibial nerve each caused progressive decreases in total tissue volume and vascular volume which were significantly less than control from 0.1 Hz to 11 Hz ($P < 0.05$).

Indicator Recovery (Fig. 3). The recovery of red cells-^{51}Cr and albumin-^{131}I was essentially complete at all stimulation frequencies of all three nerves, and, therefore, the data are reported as one group.

The recovery of ^{86}Rb decreased progressively for all three nerves as the stimulation frequency increased from 0.1 to 5 Hz. At 11 Hz the recovery continued to decrease with tibial nerve stimulation but increased with deep fibular nerve and superficial fibular nerve stimulation.

PS and CFC Changes (Fig. 4). The capillary surface area available for exchange was assessed by determining the capillary filtration coefficient (CFC) and by calculating the PS product. Tibial nerve stimulation increased PS and CFC progressively with stimulation rates up to 5 Hz and did not increase further at 11 Hz. Deep fibular nerve stimulation caused increases in PS and CFC values at 0.1 Hz and 1.0 Hz. While CFC increased at 5 Hz PS declined slightly but remained significantly above control ($P < 0.05$). At 11 Hz PS decreased to below control values and CFC decreased to the control level. When the superficial fibular nerve was stimulated the PS increased at 0.1 Hz and 1.0 Hz but then decreased at 5 Hz and 11 Hz to approximately the control levels. Stimulation of this nerve did not change the CFC from the control at any stimulation rate.

Discussion. The evidence for separate innervation of the series coupled arteries, small vessels and veins in the dog hindpaw (1, 8) would lead to the expectation that the nerves involved would individually influence blood

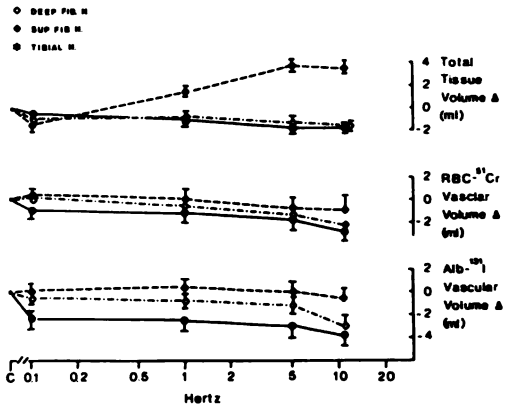


FIG. 2. Vascular and tissue volume changes with stimulation rates. Brackets indicate ±SEM.

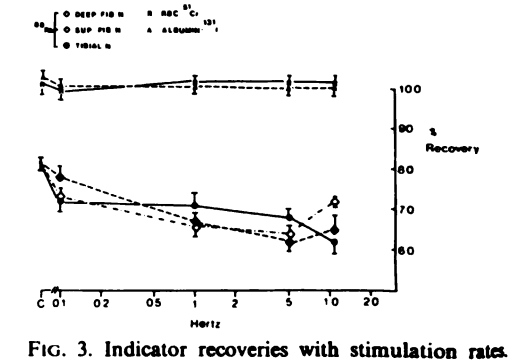
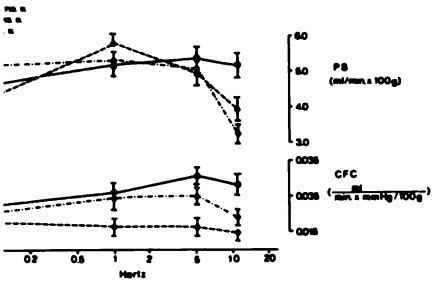


FIG. 3. Indicator recoveries with stimulation rates. Brackets indicate ±SEM.



i. 4. PS and CFC with stimulation rates. Brackets te ±SEM.

and volume distribution as well. The in this report indicate that there are ences between the effects of the nerves h also depend upon the frequency of ılation of each nerve.

perficial fibular nerve stimulation. Stim- ɔn of the superficial fibular nerve under olled pressure conditions caused de- ed blood flow consequent to increased ance. It has been demonstrated by Ham- l *et al.* (9) who measured segmental re- ıce changes that stimulation of this nerve ased small vessel resistance somewhat enous resistance to a much greater de- with no effects on arterial resistance. The in this report would support these find- The progressive increases in total tissue ne with no effect on vascular volume as ılation frequency was increased would ɔrt strong venous constriction, with con- d arterial inflow due to the relatively small vessel segment constriction. This d result in blood pooling in the small and capillaries. This pooling would el- the venous pressure resulting in pro- ively greater outward capillary filtration ıid as the stimulation rate was increased. educed downstream venous volume and ɔnsequent pooling in the small veins rently resulted in no net vascular volume ge. Therefore, the change in total tissue ne represented extravascular fluid ac- ılation. It is difficult to assess the lack of ment between the PS and CFC values. blood flow redistribution should be ted by changes in these values one d be forced to conclude that on the basis e CFC values little redistribution oc- d at any stimulation frequency although was a tendency to lower CFC at 11 Hz, ating either increased precapillary sphincter constriction or less uniform perfusion of the capillary bed. Also, the CFC may not be accurate under these conditions of large vein constriction as a smaller percentage of the venous pressure change would be transmitted to the capillaries, and therefore the CFC may be underestimated. The PS values, however, increased at 0.1 and 1 Hz and then decreased at 5 Hz, and to below control levels at 11 Hz. The increased PS values might reflect a larger extraction of Rb^{86} due to the higher outward filtration of fluid than would occur when there is little net filtration. One could expect the extraction to be proportionally greater at 5 Hz and 11 Hz if extraction is filtration dependent since outward filtration was greatest at these frequencies. Blood flow decreased to very low levels at these rates of stimulation but the extraction did not increase proportionally. It is, however, difficult to accept redistribution of blood flow away from the capillary bed when large amounts of fluid are being filtered so that edema is frankly obvious. It has also been suggested by Renkin (12) that the PS is not valid when blood flow is changing. This would be particularly true at the low flows that exist with the higher stimulation frequencies.

Deep fibular nerve stimulation. Stimulation of the deep fibular nerve increased blood flow resistance and therefore reduced blood flow as the rate increased. Vascular and tissue volume changes were small but consistent decreases in each of these parameters occurred with increasing rate of stimulation. These would presumably be due to arteriolar constriction to a small degree and to passive reduction in venous capacity consequent to the reductions in blood flow. The PS and CFC values did change reasonably uniformly. When the nerve was stimulated at 0.1, 1, and 5 Hz there were significant increases in these values indicating that flow was redistributed due to constriction of the arteriovenous anastomoses to the capillaries. However, at 11 Hz the PS and CFC values both declined markedly. This would seem to be best explained as uneven distribution of flow through capillaries as there would be little reason to expect less constriction of the A-V anastomoses at this higher frequency. Rather, one would expect more constriction of these vessels.

Tibial nerve stimulation. Stimulation of the

tibial nerve at increasing frequencies resulted in progressive increases in resistance and decreases in blood flow. The progressive declines in tissue volume and vascular volume were similar to those caused by deep fibular nerve stimulation and could be best explained on the basis of resistance vessel constriction and passive reductions in venous capacity. PS and CFC values increased progressively with stimulation rate, indicating that the A-V anastomoses were constricted strongly by these stimulations redistributing blood flow to the capillary circuits. This conclusion is supported by the report of Davis and Hammond (1) that tibial nerve stimulation resulted in redistribution of blood flow away from the digit which is rich in A-V anastomoses.

Summary. The effects of stimulation of the three nerves containing the sympathetic innervation of the vasculature of the dog hindpaw were studied during controlled pressure perfusion. Stimulation at increasing frequency of the superficial fibular nerve resulted in progressively greater edema formation, apparently due to progressively increasing venous resistance with little evidence of blood flow redistribution. Increasing frequency of stimulation of the deep fibular nerve caused constriction of the arteriovenous anastomoses resulting in blood flow redistribution to the capillary bed. Tibial nerve stimulation at all frequencies resulted in blood flow redistribution to the capillary circuit due to constriction of arteriovenous anastomoses.

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rinol-Induced Myocardial and Renal Damage in Nonarteriosclerotic (Virgin) and Arteriosclerotic (Breeder) Sprague–Dawley Rats[1] (40093)

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urinol (4-hydroxypyrazolo(3,4-d), an e of hypoxanthine, is a potent inhibhe hepatic enzyme, xanthine oxidase, atalyzes the conversion of hypoxand xanthine to uric acid. This drug has ed effectively to reduce hyperuricemia of gout or in the management of the al purine metabolism associated with diseases (1, 2). Although untreated icemia associated with gout or neolisease may induce acute renal failure ic acid nephropathy, high doses of nol *per se* will cause liver and renal because of the precipitation of oxythe relatively insoluble end-product urinol (3, 4). Because of a phylogeıtational defect, the enzyme uricase is ent in man and higher apes that when or nucleic acids are being metaboric acid accumulates instead of the ater soluble allantoin. Man's inability ze uric acid to its more soluble metabantoin, results in serum urate concenthat are ten times higher than mamhis excess urate can lead to precipif urate crystals causing debilitating ıation of the kidneys and joints, i.e., nder these conditions, allopurinol is ffective therapeutic agent.

everal years we have been investigaturally occurring hyperglycemia, hyemia, obesity, hypertension, arterio- and myocardial infarction which ocrepeatedly bred male and female rats These breeder rats are hyperuricemic male breeders are prone to develop lomerular and tubular degenerative changes as well as kidney stones (7, 8). Male breeder rats are prone to develop myocardial infarction and are less capable than female breeders of affecting myocardial repair (9, 10). De Wall *et al.* (11) have suggested that during myocardial anoxia, vital cardiac purine intermediates are lost. Because allopurinol inhibits the enzyme xanthine oxidase, they suggest that such an agent may prevent the irreversible conversion of xanthine to uric acid and thus preserve high energy nucleotides for myocardial contractual energy during acute myocardial ischemia. In this context, we were planning to treat rats with allopurinol during acute myocardial infarction to determine whether such treatment would have ameliorative effects on the usual pathophysiologic sequelae. Selye (4) and others (12) have shown that allopurinol, in large doses, can induce marked nephropathy. As a preliminary expedient, we subjected virgin males having no cardiovascular disease and breeder males known to have arterial disease to a regimen of graded doses of allopurinol to determine which dose level would be free of nephrotoxic effects in both nonarteriosclerotic virgin and arteriosclerotic breeder rats, the latter being prone to renal damage (7).

Materials and methods. Adult, male, virgin rats were used as subjects having no preexisting diabetes, hypertension, or arteriosclerosis. Repeatedly bred male rats were used as subjects with pre-existing diabetes, hypertension and arteriosclerosis. All of these animals were Sprague–Dawley rats and were between 6 and 8 months of age. The male breeder rats had sired four to five litters and had microscopic lesions only in their aortas but grossly visible plaques in their common iliac arteries (5). (The incidence of spontaneous arterial disease is so high in breeder rats that one can reliably expect to find arterial disease in these animals if they have been truly actively bred and rebred (5). In experiments of this kind,

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the presence of arterial disease is routinely confirmed by both gross and histopathologic examination). Virgin rats consistently weigh less (averaging 100 g less in body weight) than their breeder rat counterparts.

At the start of the experiment, two groups of virgin and breeder rats were segregated in the same animal quarters, i.e., virgin males ($n = 24$) and male breeders with microscopic aortic sclerosis ($n = 24$). These animals received no treatment, were fed a regular commercial rat diet (Purina) and served as baseline controls for the experimental animals. The experimental animals, virgins and breeders, were divided into three groups of 24 each and given a high (40 mg), moderate (10 mg), or low (5 mg) dose of allopurinol. The allopurinol (Zyloprim) was suspended in 0.4 ml of water and injected sc. daily for 10 days. The doses were adjusted and given according to body weight on a per 100 g basis. The experimental animals were fed the same diet as the controls and air, light, and humidity were carefully monitored.

At autopsy, blood was collected from the abdominal aorta with heparinized syringes, centrifuged (refrigerated), and assayed for creatine phosphokinase (CPK), glutamic oxaloacetic transaminase (SGOT), glutamic pyruvic transaminase (SGPT), lactic dehydrogenase (LDH), glucose, triglycerides, free fatty acids, cholesterol, and blood urea nitrogen (BUN), using the automated techniques developed for the Auto-Analyzer (Technicon Instruments Corporation, New York, NY). Serum urate levels were measured by a colorimetric method (13) and corticosterone (Cmpd. B) was measured by an automated modification of the fluorometric procedure of Guillemin, *et al.* (14). Pertinent organs from each animal were trimmed and weighed. The hearts and aortae of each rat were carefully examined for the presence or absence of cardiovascular disease. Tissues were fixed in 10% buffered neutral formalin (or glutaraldehyde) for histopathologic analyses, e.g., hematoxylin and eosin for routine analyses, Hale stain for mucopolysaccharides, von Kossa stain for calcium and frozen sections stained with Sudan black B for lipids. Analysis of variance and biostatistical analyses followed the procedures cited in Snedecor and Cochran (15).

Results. Growth, food consumption, outward appearance, and general behavior patterns gave no evidence of any toxic or untoward effects due to the allopurinol during the initial course of the experiment. After 1 week the experimental animals began to die suddenly without any premonitory signs of morbidity. Autopsy of these animals demonstrated that they had succumbed due to myocardial infarction and congestive failure. Therefore, the experiment was terminated after 10 days of treatment.

Weight changes. The final body weight of the virgin rats indicated that those treated with the higher doses of allopurinol were considerably heavier than the nontreated controls (Table I). Conversely, the breeder rats given the higher doses of allopurinol manifested a significant loss in body weight (Table I). Both virgin and breeder rats showed a marked increase in adrenal weight concomitant with equally severe thymus gland involution ($P < 0.001$) (Table I). The hearts and kidneys of allopurinol-treated animals, especially at the higher dose levels, were considerably heavier ($P < 0.001$) than the controls (Table I). There were no clear cut changes in the weight of the testes between control and experimental animals.

Serum biochemistry. Enzymes. Serum creatine phosphokinase (CPK) levels were greatly elevated in all of the allopurinol-treated animals in keeping with their high incidence of myocardial necrosis (Table II). Glutamic oxaloacetic transaminase (SGOT) and lactic dehydrogenase (LDH) levels were abnormally high in those animals given the 40 mg and 10 mg doses (Table II). Glutamic pyruvic transaminase (SGPT) levels manifested no change at any of the dose levels.

Lipids. Although the circulating triglyceride levels of the allopurinol-treated groups demonstrated no statistically significant changes, the free fatty acid and cholesterol levels were reduced in all of the experimental animals with the exception of the free fatty acid levels of virgin rats given the 40 mg dose of allopurinol (Table II). The hyperlipidemia which is characteristic of breeder vs virgin rats was present in these animals.

Glucose. The hyperglycemia, also characteristic of breeder vs virgin rats, was present in these animals (Table II). Definite hyper-

E I. GRAVIMETRIC DIFFERENCES BETWEEN NONARTERIOSCLEROTIC (VIRGIN) VS ARTERIOSCLEROTIC ıER), MALE, SPRAGUE–DAWLEY RATS TREATED WITH GRADED DOSES OF ALLOPURINOL FOR 10 DAYS.

	Rx	Final body wt (g)	Adrenal wt (mg)	Thymus wt (mg)	Heart wt (mg)	Kidney wt (mg)	Testes wt (mg)
erial disease	40 mg	409 ± 6[a] (22)	37 ± 1[a] (12)	105 ± 13[a] (11)	1369 ± 37[a] (11)	2530 ± 147[a] (11)	1847 ± 59 (11)
	10 mg	431 ± 6[a] (23)	29 ± 2[a] (12)	140 ± 11[a] (12)	1443 ± 40[a] (12)	1912 ± 105[a] (12)	1910 ± 49 (12)
	5 mg	392 ± 4[a] (20)	24 ± 1 (10)	330 ± 45[b] (10)	1282 ± 43[a] (10)	1433 ± 30 (10)	1858 ± 33 (10)
	No rx	360 ± 5 (24)	19 ± 4 (24)	410 ± 12 (24)	1030 ± 22 (24)	1460 ± 15 (24)	1860 ± 30 (24)
copic arterial ısc	40 mg	428 ± 7[a] (23)	37 ± 1[a] (12)	93 ± 10[a] (12)	1443 ± 28[a] (12)	2772 ± 108[a] (12)	1911 ± 51 (12)
	10 mg	451 ± 4[a] (23)	34 ± 2[a] (12)	133 ± 8[a] (12)	1377 ± 114 (12)	1760 ± 56[a] (12)	1901± 31 (12)
	5 mg	447 ± 6[a] (20)	22 ± 1 (10)	146 ± 20[a] (10)	1343 ± 46 (10)	1453 ± 56 (10)	1969 ± 42 (10)
	No rx	490 ± 4 (24)	23 ± 3 (24)	240 ± 26 (24)	1350 ± 25 (24)	1565 ± 30 (24)	1925 ± 30 (24)

0.001, compared to controls, i.e., no rx.
0.05, compared to controls, i.e., no rx.

a was present in all allopurinol- animals, at all dose levels, virgins and s (Table II).

l urea nitrogen. The BUN level of ed breeder rats is abnormally high as d in these animals (Table II). Allo- caused a great increase in BUN levels nsurate with the dose given. This was arly marked in the case of the virgin ıble II).

acid. Circulating urate levels were elevated ($P < 0.001$) in both virgin eder rats at the 40 mg and 10 mg dose ɔut normal or subnormal at the 5 mg vel (Fig. 1). Breeder rats become hy- emic after active and repeated breed-

costerone. Cmpd. B levels are usually lower in breeder vs virgin rats. How- reeder rats do not respond to most tuations as effectively as virgin rats. ıe virgin and breeder rats showed a increase ($P < 0.001$) in Cmpd. B n following treatment with 40 mg and f allopurinol (Fig. 2). Only virgin rats ted increased Cmpd. B levels ($P <$ n response to the 5 mg dose of allo- (Fig. 2).

and microscopic pathology. The kid- both the virgin and breeder rats dis- played grossly visible, sporadic foci of white spots and streaks in those animals treated with the highest dose of allopurinol (Table III). At the 10 mg dose levels, the renal abnormalities were no longer grossly visible but were microscopic. Animals treated with either the 40 mg or 10 mg dose of allopurinol displayed hematoxylin-positive deposits within and alongside of the medullary collecting tubules, papillary ducts and renal pelvis. The collecting tubules were frequently dilated with clusters of white blood cells and there were frequent foci of stone formation (Fig. 3). The renal cortices of these animals displayed foci of calcific or von Kossa positive material and glomerular hyperplasia. Breeder rats which manifest considerable renal damage including kidney stones, spontaneously, showed definite exacerbation of their usual pattern of renal pathology after treatment with the 40 and 10 mg dose of allopurinol (Table III). At the 5 mg dose level, none of these changes were apparent in the virgin, control rats. Similarly, the hydronephrosis, white blood cell infiltration, and deposition of basophilic material attributed to allopurinol did not appear in breeder rats treated at the 5 mg dose level. Only the usual glomerular damage, tubular colloid and kidney stone formation which develops sponta-

TABLE II. DIFFERENCES IN SERUM BIOCHEMISTRY BETWEEN NONARTERIOSCLEROTIC (VIRGIN) VS ARTERIOSCLEROTIC (BREEDER), MALE, SPRAGUE-DAWLEY RATS TREATED WITH GRADED DOSES OF ALLOPURINOL FOR 10 DAYS.

	Rx	CPK (μmol/l)	SGOT (μmol/l)	SGPT (μmol/l)	LDH (μmol/hl)	Trigly. (mg%)	F. Fatty a. (mEq/l)	Chols. (mg%)	Glucose (mg%)	B.U.N. (mg%)
Virgins	40 mg	240 ± 8[a] (11)	186 ± 6[a] (11)	38 ± 2 (11)	324 ± 28[a] (11)	72 ± 10 (11)	425 ± 15[a] (11)	97 ± 11[b] (8)	151 ± 5[a] (11)	81 ± 7[a] (11)
	10 mg	133 ± 6[a] (10)	175 ± 4[a] (10)	35 ± 1 (10)	192 ± 18 (10)	108 ± 6 (10)	101 ± 8[a] (10)	97 ± 6[b] (10)	149 ± 5[a] (10)	28 ± 1[a] (10)
No arterial disease	5 mg	160 ± 5[a] (9)	81 ± 2 (9)	40 ± 1 (9)	61 ± 5 (9)	89 ± 8 (9)	126 ± 18[a] (9)	83 ± 4[b] (9)	176 ± 4[a] (9)	26 ± 1[a] (9)
	No rx	105 ± 3 (24)	116 ± 4 (24)	42 ± 4 (24)	180 ± 9 (24)	80 ± 4 (24)	286 ± 31 (24)	124 ± 6 (24)	115 ± 9 (24)	14 ± 2 (24)
Breeders	40 mg	250 ± 16[a] (10)	195 ± 19[a] (10)	38 ± 2 (10)	323 ± 32[a] (10)	73 ± 7 (10)	449 ± 28 (10)	77 ± 3[a] (10)	154 ± 4[a] (10)	84 ± 17[a] (10)
	10 mg	180 ± 11[a] (12)	175 ± 3[a] (12)	48 ± 1 (12)	224 ± 21[a] (12)	67 ± 5 (12)	248 ± 8[a] (12)	73 ± 2[a] (12)	150 ± 5[a] (12)	33 ± 1[a] (12)
Microscopic arterial disease	5 mg	160 ± 5[a] (9)	77 ± 3 (9)	40 ± 2 (9)	65 ± 7 (9)	66 ± 5 (9)	169 ± 17[a] (9)	81 ± 3[a] (9)	167 ± 5[a] (9)	26 ± 1 (9)
	No rx	125 ± 5 (24)	138 ± 6 (24)	49 ± 3 (24)	170 ± 8 (24)	110 ± 5 (24)	410 ± 38 (24)	205 ± 24 (24)	249 ± 8 (24)	26 ± 2 (24)

[a] $P < 0.001$, compared to controls, i.e., no rx.
[b] $P < 0.05$, compared to controls, i.e., no rx.

neously in breeder rats was present (Tabl III).

Male breeder rats are prone to develo myocardial infarction spontaneously (Tabl III). Administration of 10 mg and 40 mg o allopurinol caused a definite exacerbation o both the incidence and severity of myocardia infarction in breeder rats (Table III). Of par ticular interest was the appearance of my ocardial infarction in non-arteriosclerotic virgin rats treated with 40 mg and 10 mg o allopurinol (Table III). The myocardial dam age in virgin rats was not as severe as i breeder rats; there were scattered foci of ne crotic muscle with little white blood cell in filtration within the left and right ventricle (Fig. 4). There were no changes which coul be ascribed to allopurinol treatment pertinen to the incidence, severity, or morphologi composition of the arterial lesions which ap pear spontaneously in breeder rats.

The livers of breeder rats are consistentl infiltrated with lipid (Table III). As in th case of the heart and kidney, the high dose of allopurinol caused a definite worsening o this naturally-occurring, fatty infiltration o the liver in breeder rats and the *de nov* appearance of hepatic lipid in virgin rat (Table III). Particularly striking was the ap pearance of scattered foci of necrosis (Fig. 5 in the livers of both virgin and breeder rat given the high doses of allopurinol (Tabl III). Similarly, breeder rats characteristicall exhibit degranulation of their insulin-produc ing cells commensurate with their hypergly cemia (Tables II and III). Therefore, it wa difficult to ascertain whether treatment witl allopurinol caused any worsening of the bet cell degranulation in breeder rats. Howeve there was a definite pattern of *de novo* bet cell degranulation (Table III) and hypergly cemia (Table II) in the virgin rats at all dos levels of allopurinol.

The thymi of all of the treated animal were greatly involuted. The adrenal cortice were hyperplastic and exhibited extensiv lipid depletion from the inner cortical zone The testes showed no evidence of abnormal ity.

Discussion. These findings demonstrat that allopurinol, the potent inhibitor of th hepatic enzyme xanthine oxidase, can pr duce definite myocardial, renal, and hepati

toxic effects if given at high dose levels in the rat. The nature of the sudden demise of these animals and our finding of myocardial infarction and congestive heart failure and the

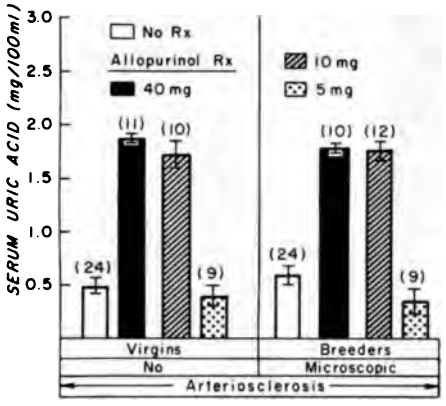

FIG. 1. Changes in serum uric acid levels in nonarteriosclerotic (virgin) vs arteriosclerotic (breeder), male, Sprague–Dawley rats given varying doses of allopurinol for 10 days. The height of each column depicts the mean ± SE; ( ) = number of samples.

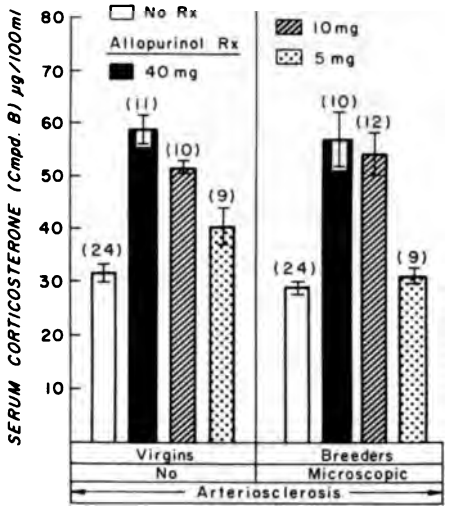

FIG. 2. Changes in serum corticosterone levels in nonarteriosclerotic (virgin) vs arteriosclerotic (breeder), male, Sprague–Dawley rats given varying doses of allopurinol for 10 days. The height of each column depicts the mean ± SE; ( ) = number of samples.

TABLE III. PERCENT INCIDENCE OF PATHOLOGIC CHANGES INDUCED BY GRADED DOSES OF ALLOPURINOL IN NONARTERIOSCLEROTIC (VIRGIN) VS ARTERIOSCLEROTIC (BREEDER), MALE, SPRAGUE-DAWLEY RATS AFTER 10 DAYS OF TREATMENT.

	Rx	Heart	Kidney	Liver	Pancreas
Virgins	40 mg	44% + m.i.[a]	100% + nephrosis[b]	15% patchy necrosis[c] +	61% beta cell + degran[d]
	10 mg	26% + m.i.	74% + nephrosis	8% patchy necrosis +	59% beta cell + degran.
No arterial disease	5 mg	0%	0%	0%	64% beta cell + degran.
	No rx	0%	0%	0%	0%
Breeders			50% +		
	40 mg	55% ++ + m.i.	50% +++ nephrosis	26% patchy necrosis ++	84% beta cell ++ degran.
	10 mg	2% +++ 60%m.i. +	75% + nephrosis	18% patchy necrosis ++	86% beta cell ++ degran.
Microscopic arterial disease	5 mg	16% + m.i.	20% + nephrosis	78% fatty liver ++	85% beta cell ++ degran.
	No rx	18% + m.i.	18% + nephrosis	81% fatty liver ++	91% beta cell ++ degran.

[a] Myocardial infarction.
[b] Glomerular damage, tubular dilatation, hydronephrosis, wbc infiltration and/or stone formation. [c] Foci of necrosis and infiltration of lipid.
[d] Aldehyde fuchsin positivity of granules.
+++ = severe, ++ = moderate, + = slight

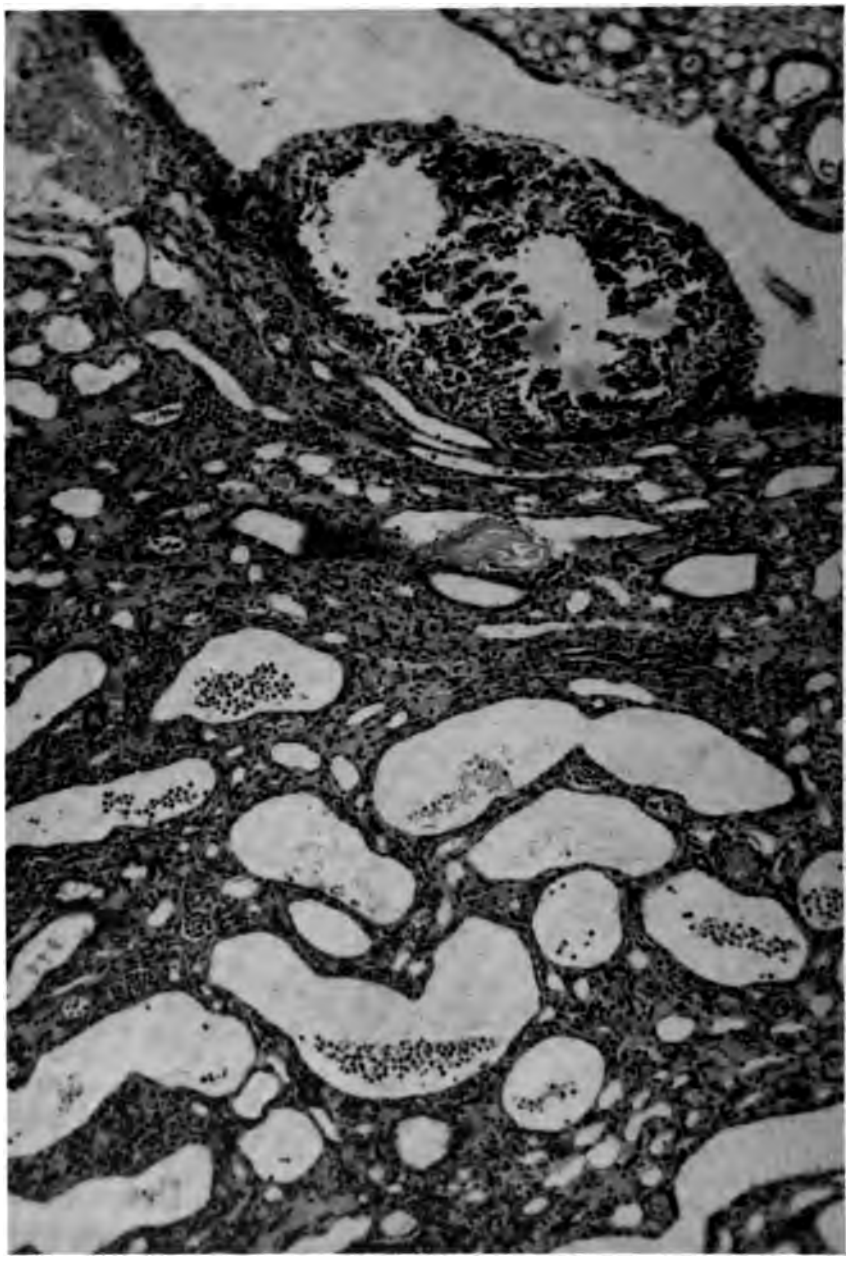

FIG. 3. Kidney of a virgin male, Sprague-Dawley rat treated with 40 mg of allopurinol daily for 10 days. collecting tubules are greatly dilated or hydronephrotic and contain abundant wbc's. The large, ovoid body embed in the transitional epithelium lining the renal pelvis is a urate stone. H and E, ×100.

greatly elevated serum CPK and LDH levels, attests to myocardial toxicity as the immediate contributing cause of death. Although high-energy nucleotides are essential for myocardial contractural energy (11), an excess of purines, as in this experiment, would interfere with myocardial function. The hea body weights of allopurinol-treated vi rats we believe is a reflection of their hy thorax condition or congestive heart fail whereas the reduced body weights of bre rats reflects their greater susceptibility tow

ɔ. 4. Myocardium of the same animal described in Fig. 3 showing the extensive myocardial necrosis (light grey in photo) which accompanies the triad of myopathy, nephropathy (Fig. 3), and hepatic damage (Fig. 5) which ıpanies an overdose of allopurinol. H & E, ×125.

oxicity of the high doses of allopurinol, :atabolism. Further, we believe that the ıy hepatic necrosis caused an exacerba- ɔf the congestive heart failure and neph- ıicity because of the reduced ability of ver to conjugate steroids and other me- ites, especially the extra amounts of al- lopurinol. The hyperplasia of the adrenal glands, their increased weight and lipid depletion as well as increased Cmpd. B secretion, coupled with marked thymus gland involution, all attest to the severe stressful nature of this regimen of treatment.

The elevation of serum SGOT levels is in

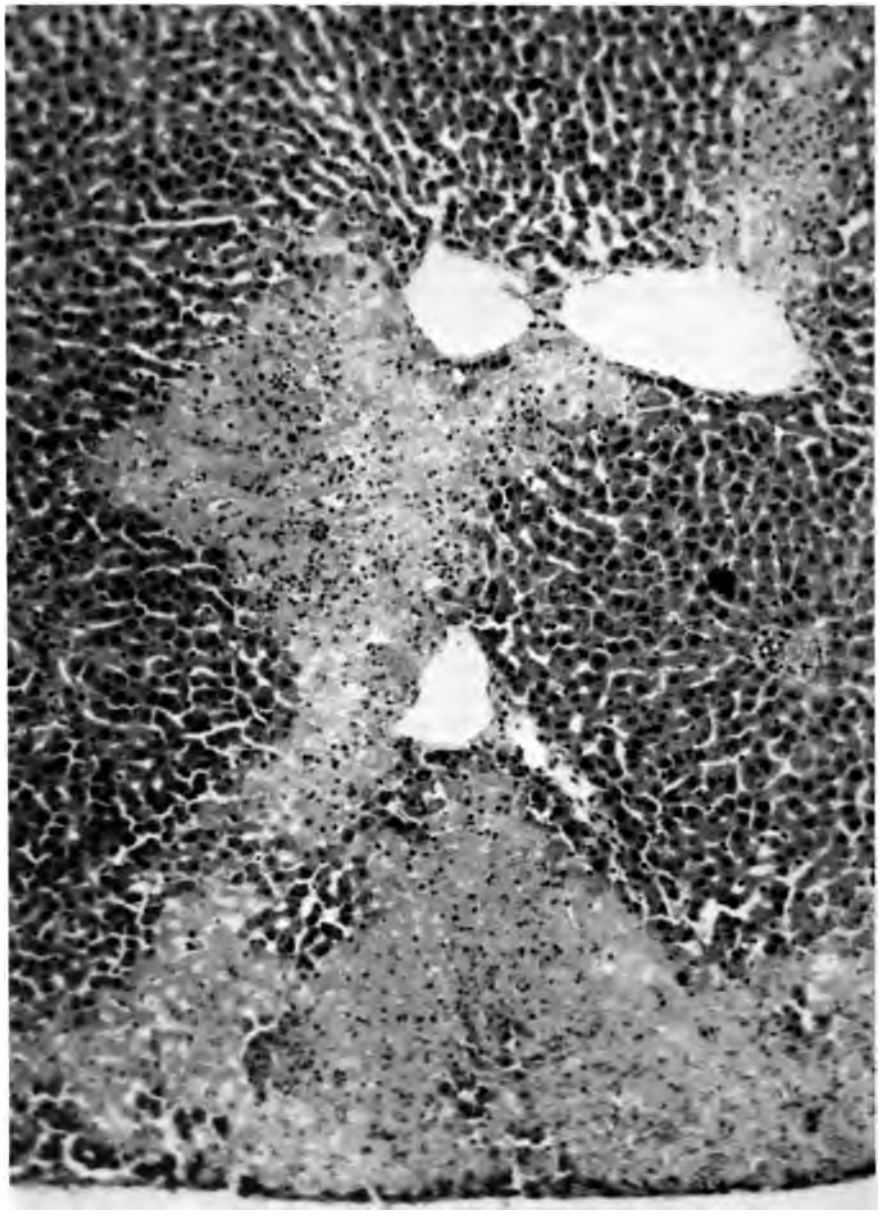

FIG. 5. Liver of the same animal shown in Figs. 3 and 4, showing the large and extensive foci of hepatic necr which attends the use of high doses of allopurinol. (The fatty infiltration of the liver which is also characteristic allopurinol overdose is not visible in this particular section). H and E, ×125.

keeping with the severe liver damage but the lack of change in SGPT levels and reduction in circulating lipid levels, despite the fatty liver condition, is difficult to reconcile. The marked hyperglycemia in both virgin and breeder rats treated with allopurinol at all dose levels, coupled with the consistent beta cell degranulation, suggests some insu blocking effect of this drug either at the p ripheral or beta cell level, or the presence excess uric acid metabolites had an alloxa like effect, i.e., uric acid yields alloxan wh oxidized (16).

The exceptionally high BUN levels in

gin and breeder rats given the high dose of allopurinol are indicative of the severe nephrotoxic and hepatotoxic effects of this drug in high doses (3, 12, 17). It is paradoxical to find hyperuricemia in animals given larger doses of allopurinol. These high levels of allopurinol could react colorimetrically and give falsely high uric acid levels. However, we could not demonstrate such interference. The high urate levels in these animals may have been due to impaired renal excretion primarily and to decreased hepatic metabolism secondarily. Allopurinol is cleared rapidly but oxypurinol, its immediate metabolite, is reabsorbed by the kidney tubule and is excreted slowly (3). It is possible that the grossly visible white streaks and appearance of kidney stones in animals given the large doses of allopurinol are either depots of excess allopurinol and its metabolites, e.g., oxypurinol, or admixtures of calcium and urate.

It is of interest that although the arteriosclerotic breeder rats showed definite exacerbation of most of their naturally-occurring pathophysiologic stigmata, e.g., myocardial infarction, there was no apparent change in the nature of their arterial disease despite the expression of severe toxicity in key organs.

Summary. Non-arteriosclerotic (virgin) and arteriosclerotic (breeder), male Sprague–Dawley rats were treated with high (40 mg), moderate (10 mg) and low (5 mg) doses of the potent xanthine oxidase inhibiting agent, allopurinol. After 10 days of treatment, the animals began to die suddenly due to myocardial infarction, hepatic necrosis and nephrotoxic damage. Serum enzyme levels, e.g., CPK, SGOT and LDH, were abnormally elevated in both the myocardial infarct prone breeder rats and the otherwise healthy, virgin rats commensurate with a high incidence of heart damage in both the virgin and breeder rats. Although the high dose of allopurinol caused fatty infiltration of the liver, the experimental animals were hypolipidemic. The severity of islet beta cell degranulation paralleled the degree of hyperglycemia in all animals given allopurinol. Elevated BUN levels, high circulating urate levels, hydronephrosis, and urate stones were commensurate with the dose of allopurinol administered. Increased adrenal weight, hyperplasia, lipid depletion, and abnormally high circulating Cmpd. B levels, along with marked thymus gland involution, attested to the severe noxious effect of overdose with allopurinol. It is suggested that the unusual appearance of myocardial damage in the nonarteriosclerotic virgin and myocardial infarct-prone arteriosclerotic breeder rats is primarily related to the cardiotoxic and nephrotoxic effects of large doses of allopurinol and secondarily to its hepatotoxic effects.

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Inhibition of Bovidae Oocyte Meiosis by Serine Protease Inhibitors (40094)

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It has been known for a number of years that various proteases can induce meiosis in the eggs of marine forms (1, 2). A recent report by Peaucellier (3) indicates that two serine proteases in the digestive tract of the polychaete annelid, *Sabellaria alveolata* initiate meiotic maturation in shed oocytes. The suggestion of Richert and Ryan (4, 5) that serine proteases are involved in the activation of rat ovarian adenylate cyclase by LH prompted the present investigation into a potential role of serine proteases in the re-induction of meiotic maturation in female mammalian systems.

Materials and methods. The method of Jagiello (6) for *in vitro* maturation of Bovidae oocytes was used as the biological assay system. Bovidae oocytes were removed from medium sized follicles and immediately placed into McCoy's media with additives as noted in Tables I–III. They were matured *in vitro* in a constant 5% CO_2/air high humidity atmosphere at pH 7.2 for 24–26 hr. At the end of the incubation the oocytes were prepared for analysis by a modification of the method of Tarkowski (7) and stained with 1% toluidine blue in resin. The attainment of normal first or second meiotic metaphase as scored by cytogenetic examination with a Zeiss 100× bright light objective was considered the end point indicating successful germinal vesicle breakdown and meiotic progression. Cow oocytes, which have a high percentage of maturation *in vitro* in 24–26 hr were used for studying inhibition and conversely, ewe oocytes, which have a moderate percentage, were used for studying meiotic initiation.

The specific site-directed synthetic inhibitors for trypsin, *N-p*-tosyl-L-lysine chloromethyl ketone (TLCK); chymotrypsin, *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Sigma Chemical Company, St. Louis, MO) and elastase, acetylalanyl alanyl prolyl alanine chloromethyl ketone (prepared by Dr. James Powers, Georgia Institute of Technology, Altanta, GA), the trypsin substrate analog, *N*-tosyl arginine methyl ester HCl (TAME) (Sigma, St. Louis, MO), the inhibitor of esterases involved in the phosphorylation of serine residues in their active centers, phenyl-methyl sulfonyl fluoride (PMSF) (Schwarz Mann, Orangeburg, NY), and the specific chymotrypsin inhibitor, diphenyl carbamyl chloride (DPCC) (Eastman Kodak Co., Rochester, NY) were studied. Solutions of these agents were freshly prepared in 95% ethanol or DMSO and diluted wtih media so that the final assay concentrations of alcohol did not exceed 0.025% ethanol or 1% DMSO. Inhibitors in concentrations indicated on Table I were added just prior to the incubation. The natural serine protease inhibitors serum α_1-antitrypsin (National Fractionation Center–American Red Cross, Washington, D.C.), soybean trypsin inhibitor (Worthington Biochemical Corp., Freehold, NJ), and leupeptin (obtained with the courtesy of Dr. Lowell Greenbaum, Columbia University College of Physicians and Surgeons) were freshly dissolved in media and tested with oocytes at final concentrations noted in Table II. The effects of the three pancreatic serine proteases, trypsin (TRYPTAR) (Armour Pharmaceutical Co. Chicago, IL), chymotrypsin (Worthington Biochemical Corp.) and elastase (Elastin Products, St. Louis, MO), and of a crude human sperm acrosin (prepared by Dr. Wylie Hembree, Columbia University College of Physicians and Surgeons) were dissolved in media and studied in the concentrations noted in Table III.

Results. As seen in Table I, all of the protease inhibitors tested, except the elastase inhibitor, affected cow oocyte maturation *in vitro* ($P < 0.005$) in a dose dependent manner. By cytologic examination the inhibited nuclei

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ABLE I. EFFECTS OF SYNTHETIC PROTEASE NHIBITORS ON COW OOCYTE MATURATION.[a]

ditive	Final concentration (m*M*)	Oocytes Total	number matured	%
	0	64	56	88
K	0.001	49	36	73
	0.01	38	21	55*
	0.1	69	29	42*
	1.0	101	43	43*
	5.0	123	21	17*
	0	99	79	80
K	0.001	54	33	61
	0.01	80	61	76
	0.1	74	44	59*
	1	80	20	25*
	5	135	17	13*
	0	79	64	81
F	0.001	40	25	63
	0.01	81	51	63
	0.1	71	37	52*
	1.0	40	18	21*
	5.0	87	13	15*
APACK	0.00002	92	65	70
	20	50	44	88
	0	74	61	82
E	0.01	41	31	76
	0.1	47	37	79
	1.0	99	45	45*
	5.0	78	31	40*
C	0.01	60	40	67
	.1	52	28	54*
	1.0	34	6	18*
	20.0	39	0	0*

All cultures were carried out in McCoy's 5A medium
nd Island Biological Co.)
$^{*}P < 0.005$ inhibition.

BLE II. EFFECTS OF NATURAL INHIBITORS ON COW OOCYTE MATURATION.

dditive	Inhibitor final concentration per ml	Oocytes Total	Number matured	%
	0	118	97	82
ntitrypsin	5 mg	74	46	62
	10 mg	94	0	0
bean tryp-	5 mg	80	56	70
inhibitor	10 mg	54	34	63
eptin	10 ng	50	34	68
	10 mg	91	70	76

he presence of each agent had emerged
the germinal vesicle stage and had
hed the stage of late condensed chroma-
The approximate concentrations required
hibit maturation of 50% of the oocytes relative to the controls were 0.1 m*M* TLCK and PMSF, 0.3 m*M* for TPCK, 3.0 m*M* for TAME and 0.1 m*M* for the chymotrypsin inactivator DPCC. These approximate potencies were obtained from a logit-log plot of the data in Table I. Solvents alone were without effect. The natural trypsin inhibitor α_1-antitrypsin was dramatically inhibitory (Table II) at a concentration of 10 mg/ml. Attempts to activate additional meiotic activity above control in ewe oocytes with the pancreatic serine proteases trypsin, chymotrypsin and elastase (Table III) were unsuccessful. However, crude human sperm acrosin was quite *inhibitory* at concentrations of 1–100 μg/ml ($P < 0.005$).

Discussion. The inhibition of cow oocyte meiotic maturation by specific inhibitors of serine proteases documented in the present study suggests that a protease or proteases with a similar molecular conformation play a role in the resumption of mammalian meiosis. Since the nuclei attain the early stages of germinal vesicle breakdown and chromatin condensation, it would appear that the initial process of meiotic resumption occurs, but fails to progress when protease activity is inhibited.

Attempts to induce a stimulatory effect on *in vitro* ewe meiosis with pancreatic serine proteases failed, even in concentrations far in excess of those shown to activate the luteal cell adenyl cyclase system (4, 5). Peaucellier (3) also was unable to mimic the meiosis

TABLE III. EFFECTS OF SERINE PROTEASES ON EWE OOCYTE MATURATION.

Additive	Final concentration (μg/ml)	Oocytes Total	Number matured	%
None	0	114	86	75
Trypsin	3	78	62	79
	10	78	58	74
	25	32	24	75
	250	91	65	71
α-Chymotrypsin	3	41	31	76
	10	52	38	73
	25	39	28	72
	250	43	27	63
Elastase	25	37	24	65
	250	84	57	67
Acrosin	1	62	34	55*
	10	70	34	49*
	100	52	14	27*

* = $P < 0.005$.

inducing effects of the *Sabellaria* digestive enzymes by equimolar concentrations of commercially available preparations. A different substrate specificity between the pancreatic and ovarian proteases may account for these findings or may be related to an inability of exogenous proteases to gain access to the proper oocyte substrate. The inhibitory effect of the crude human sperm acrosin may be due to acrosin itself or to other contaminating sperm enzymes.

Assays for trypsin-like activity by the technique of Erlanger *et al.* (8) in pools of 200–1000 cow oocytes did not reveal any enzymatic activity. The fact that meiotic maturation is blocked by both trypsin (TLCK, TAME and α_1-antitrypsin) inhibitors as well as chymotrypsin (TPCK, DPCC) inhibitors suggests either that there is a cascade of proteases or there is a single protease with unique properties. A protease from rat liver has been described that is inhibited by both trypsin and chymotrypsin inhibitors (9). This liver protease also has a highly restricted substrate specificity. The failure to demonstrate protease activity in cow oocytes may therefore be due to an inappropriate substrate.

Bovine and ovine fluids from medium-sized ovarian follicles were found to have trypsin inhibitory activity of 1.70 mg and 1.62 mg trypsin inhibited per ml fluid, a finding consonant with the report of meiotic inhibition by Bovidae follicular fluid (10) and th data in the present report. The mechanism o action of the specific serine proteases in th re-induction of mammalian oocyte meiosis i being explored in further experiments.

Summary. Specific inhibitors of serine pro teases have been shown for the first time t inhibit meiotic progression in mammalian oo cytes, suggesting involvement of these en zymes in the process of maturation.

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uction of Malformations by Actinomycin D at Mid-Pregnancy in the Rat and Protection by L-Thyroxine[1] (40095)

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ntibiotic actinomycin D is a potent ıic agent in laboratory animals (1), ıcks DNA-directed synthesis of mes- m)RNA upon administration (2). iven by intraperitoneal injection to : rats before mid-gestation, actino-) caused intra-uterine growth retar- ıd abnormal development of several stems (3–5); no malformations were l, however, when treatment was be- r day 10 of pregnancy. The work of *t al.* (6) suggested that subcutaneous . of the drug might extend its range ın, and preliminary experiments ındeed that actinomycin administra- the subcutaneous route on day 12 evere edema, club foot, crook tail, mphalos in the offspring. In subse- ţperiments, reported below, actino-) was injected alone and in combi- ıith thyroxine, in the hope that the atment might ameliorate the terato- ţion of the drug: thyroxine has been) stimulate nucleic acid synthesis (7) ırevent the growth-inhibitory effects ɔmycin D by combining with it (8). the fetal rats, obtained by Cesarian n day 21 of pregnancy, were kept for s to study the long-range effects of ment.

ials and methods. Thirty-six pregnant re separated randomly into four Group I, 12 rats, received subcuta- 200 μg/kg of actinomycin D[3] on day 12 of pregnancy; the day of finding sperm in the vagina was considered day 0. Group II, eight rats, received subcutaneously 200 μg/kg of thyroxine[4] on day 12 followed immediately by the same dose of actinomycin D. Group III, eight rats, was injected subcutaneously with 200 μg/kg of thyroxine on day 11 of pregnancy; the rats received the same amount of actinomycin D on day 12. Group IV consisted of eight uninjected rats. Other uninjected pregnant rats, scheduled to deliver at the same time as the rest, were kept in readiness to serve as foster mothers for some offspring of the experimental group.

The rats were housed under natural conditions of light and darkness in a temperature-controlled room and provided with stock diet[5] and distilled drinking water *ad libitum*. The young were removed under ether anesthesia on day 21 of pregnancy, weighed and examined for gross abnormalities. Some litters were killed, fixed and subsequently examined for abnormalities by razor blade sectioning. Selected members of other litters were placed with foster mothers and were raised to age 6 months.

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ent of USPHS Fellowship 5-F1-GM-16, 474- ıt address: Department of Otolaryngology, n University School of Medicine, 517 S. Eu- ıuis, MO 63110.

omycin, obtained from Merck, Sharpe and Dohme, Rahway, NJ. The drug was dissolved in 10 ml of propylene glycol (1,2-propane diol) and brought with distilled water to final volume of 625 ml and concentration of 40 μg/ml. The final concentration of propylene glycol was 3 mg/ml, and the amount administered in this study was approximately one-tenth the dose having possible teratogenic effects in mice (9). No evidence exists to implicate propylene glycol as a teratogen in rats.

[4] L-Thyroxine, obtained as sodium salt, pentahydrate, from Aldrich Chemical Co., Inc., Milwaukee, WI, and administered as an alkaline solution. The hormone and actinomycin D were injected at adjacent sites between the shoulder blades.

[5] The diet consisted of 67.5% ground whole wheat, 15% casein, 7.5% skim milk powder, 0.75% NaCl (sufficient KI added to furnish 1 μg iodine per g of diet), 1.5% $CaCO_3$, 6.75% hydrogenated vegetable oil, and 1% fish oil. Fresh lettuce was supplied twice weekly.

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TABLE I. EFFECTS OF ACTINOMYCIN D ALONE AND IN COMBINATION WITH L-THYROXINE ON REPRODUCTIVE PERFORMANCE OF LONG-EVANS RATS AND ON SURVIVING YOUNG.

Group[a]	Treatment schedule (dose/kg of body weight)	Treatment schedule (day of pregnancy)	Average no. of implants	Surviving young (%)	Average wt of survivors (g)	Abnormal survivors (%)
I (12)	200 μg actinomycin	12	13	63	5.5	14[b]
II (8)	200 μg actinomycin + 200 μg thyroxine	12 12	12	86	5.8	2[c]
III (8)	200 μg actinomycin + 200 μg thyroxine	12 11	11	85	5.9	0
IV (8)	—	—	12	92	5.7	0

[a] Number of animals in parenthesis. They were killed on day 21 or pregnancy (day of finding sperm = day 0) and delivered of their young by Cesarean section.

[b] Eleven survivors had club feet, one had exomphalos, and two were severely stunted and weighed less than 4 g at delivery.

[c] Mild edema.

Results. All rats gained weight in the course of the experiment, but only those in Group III matched the growth rates of Group IV animals. All pregnant rats had live young. None of the treatments affected the average number of implantation sites or the average weight of surviving young at term (Table I), but only 63% of the implants in Group I survived to day 21 of pregnancy. Survival in Groups II and III was about equal and much improved over that in Group I. Fourteen percent of survivors were abnormal in Group I, whereas only 2% were abnormal in Group II, and there were no malformed young in Group III. Of 14 abnormal young in the first group, 11 had club foot, one had exomphalos and two were severely stunted. The only abnormality noted in some Group II animals was slight edema which disappeared within a day.

All of the male and female offspring in Groups II, III, and IV selected for observation survived to age 6 months, when they were killed. On the other hand, only 70% of males and 50% of females in Group I survived that long. Deaths in this group occurred within the first 90 days of life; no cause of death was found at autopsy. Pregnancy was uneventful in the four Group I females that were mated with littermates, and newborn appeared normal in all respects.

Discussion. The data presented in this study demonstrate that choice of a different route of administration can extend the teratogenic time range of actinomycin D to day 12 or beyond. Abnormalities most often encountered were club foot, exomphalos, generalized edema, and overall stunting of growth.[6] There was also a significant elevation in fetal mortality, but survivors were of normal size and proportions, except as noted. The teratogenic effects were completely suppressed by prior treatment of the mothers with thyroxine, and embryolethal effects were reduced significantly. Concurrent injection of thyroxine and actinomycin D caused a similar reduction of lethal effects and prevented permanent defects. Actinomycin D seems to bind more readily to differentiating cells than to cells which have already differentiated (11), and it may be conjectured that thyroxine administered on day 11 of development prevented the teratologic action of the drug by accelerating development of the embryo past the state of susceptibility. Alternatively, the hormone by augmenting available nuclear binding sites may have neutralized the cytotoxic and cytostatic action of the drug; both in the chick (12) and hamster (13), DNA administered together with actinomycin D reduced the embryolethal and teratogenic effects of the antibiotic. However, neither hypothesis can explain adequately our finding that thyroxine given concurrently with actinomycin was almost as effective in protecting mother and fetus as was its injection the day before. Kim *et al.* (8) reported that thyroxine formed a complex with actinomycin D similar to that

[6] Spontaneously occurring congenital malformations in Long-Evans rats of our colony are exceedingly rare (10), and none was seen in control rat litters of this study.

formed by the antibiotic with deoxyguanosine. Whereas actinomycin alone completely inhibited growth of bacterial cultures, growth was normal when hormone was added to the medium. Formation of such a complex may also have resulted from an interaction of actinomycin D and thyroxine in this study, and its inability to enter the cell in toxic quantities could account for the minimal teratogenic effect of the drug.

Summary. The embryolethal and teratogenic effects of actinomycin D were studied in Long-Evans rats. Subcutaneous administration extended the teratogenic time range of the drug beyond that previously identified by intraperitoneal injections; a single dose (200 μg/kg body weight) given on day 12 of pregnancy caused club foot, generalized edema, exomphalos, and growth retardation in 14% of surviving young. When equal amounts of L-thyroxine were injected concurrently with actinomycin D on day 12 of development, the effects of actinomycin on the mothers were ameliorated and abnormalities in the young were strikingly reduced. When injected one day previous to treatment with actinomycin D, thyroxine prevented fetal malformations. These results may be interpreted on the hypothesis that the hormone forms a harmless complex with actinomycin D.

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Characterization of Transplantable Myelomonocytic Leukemia WEHI-3B in Syngeneic BALB/c Mice[1] (40096)

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Clinical trials have shown (1–3) that remission and survival in acute myelomonocytic leukemia (AMML) is increased significantly by immunotherapy. Despite these important clinical findings few well characterized transplantation models of the disease in inbred mice are in use for the analysis of basic immunologic mechanisms. By employing an appropriate model it should be possible to characterize the relative malignancy of the major cell populations that comprise the disease, their relative immunogenicity, whether a protective immune response is serum or cell mediated, and the effects of drugs and immunopotentiating agents on the course of the disease. We are particularly interested in determining whether the immune mechanism recognizes with equal avidity the diverse malignant cell populations that comprise the disease. Warner, Moore and Metcalf (4) originally described the occurrence of AMML in BALB/c mice and derived four sublines from the original parental tumor (WEHI). The A and D sublines were chloromas. The 3B and 3C lines appeared to approximate AMML in humans. We report here the basic characteristics of a transplantation model of WEHI-3B AMML.

Methods. Mice. Inbred BALB/cwm mice (referred to as BALB mice hereafter) were obtained from our breeding stocks. Mice were 6–8 weeks old when used. The lineage of the strain and hematologic properties of the mice were described (5). Female mice were used for all experiments. *WEHI-3B line of AMML.* We obtained the WEHI-3B line from Dr. D. Metcalf of The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. On receipt the subcutaneous tumor was transplanted immediately into our strain of mice. The methods used to develop the subcutaneous (SQ), splenic (SP) and intraperitoneal (IP) sublines are described under *Results.*

Balanced salt solution. WEHI cells are sensitive to the salt composition and pH of media. A balanced salt solution (MBSS) was used that was optimum for maintaining cell viability. MBSS contained per L of double distilled water: 0.14 g $CaCl_2$; 8.0 g NaCl; 0.4 g KCL; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.2 g $MgCl_2 \cdot 6H_2O$; 0.06 g KH_2PO_4; 0.24 g $Na_2HPO_4 \cdot 2H_2O$; 0.01 g phenol red (Allied Chem. Corp., NY) and 4.5 g Hepes buffer (Schwarz-Mann, Orangeburg, NJ). The solution was sterilized by filtration through a 0.22 μfilter and the pH adjusted to 7.2.

Dose-response assays. Details are presented under RESULTS. The method of Skipper, Schabel and Wilcox (6) was used to determine whether a single viable cell of the IP subline was lethal to mice.

Histopathologic methods. Mice (3/group) inoculated iv or ip with 10^4 viable cells were killed at selected time intervals. Control mice (4/group) received injections of MBSS in place of the cell suspensions and were killed and examined at the end of the experiment. Samples of the thymus, spleen, mesenteric lymph nodes, heart, mediastinum, lung, kidney, adrenal gland, femur (bone marrow), genitourinary organs, gastrointestinal tract, and brain were taken for examination. Tissues were fixed in 10% (v/v) formalin–saline, paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Cell blocks were prepared by centrifugation of cell suspensions, fixed in Bouin's solution, and similarly embedded, sectioned and stained.

Hematologic methods. Each of 55 mice received a single ip injection of 10^4 viable cells contained in 1 ml of MBSS. Control mice (14

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ıls) received an ip injection of 1 ml of
; and were killed either on the day of
ion or 18 days later. Methods were de-
d in detail (5). Most animals were bled
ɔnce. If mice were bled twice the first
ng was done on day 13, followed by a
d bleeding on day 20 or 21. Peripheral
was obtained by cutting 1–2 mm off
il and then letting the blood flow freely.
nens taken 30–60 sec later were col-
directly into hematologic pipettes and
inized microhematocrit tubes. Total
ifferential leukocyte counts were made
e method described by Wintrobe (7).
ıeral blood samples were diluted 1:20
ematologic pipette with Turk's solution
glacial acetic acid (v/v), 1% Gentian
(v/v), in distilled water). Erythrocytes
allowed to lyse for 5 min and the leu-
ɛs then counted in a hemocytometer.
ɛrcent distribution of leukocyte types
letermined by counting 100 cells on
ıt stained films. The microtechnique
ɛd for hematocrit determinations. Hep-
ɔoated capillary tubes were filled ap-
nately halfway and sealed at both ends.
l tubes were placed in microhematocrit
head and centrifuged for 5 min at 4000
The percent of packed cells was deter-
l by use of a microhematocrit tube
r.

ting efficiency of cell lines. Standard pro-
ɛs were used for passing the tumor cell
or efficiency of plating tests: Mice were
lated sq with 2.25×10^5 cells of the SQ
umors were harvested 17–21 days later.
oculum (ip) of 10^5 IP cells was used;
s cells were harvested 14–16 days later.
ıe SP line 10^7 tumor cells were injected
nor cells were harvested approximately
/s later. Under these conditions the cell
ations of each transplanted line con-
l the greatest proportion of tumor cells
ermined by microscope observation; vi-
' was 85–90%. Mondisperse prepara-
of cells were suspended to the desired
ıtration in RPMI 1640 medium (Grand
. Biological Co., Grand Island, NY)
ning 10% fetal calf serum (inactivated
for 30 min). To plate cells serial tenfold
ɔns were made in double strength RPMI
ı medium containing 20% inactivated
alf serum, 200 U/ml of penicillin and 200 μg/ml of streptomycin. Difco agar (Difco Laboratories, Detroit, MI) was made up in double distilled water to a final concentration of 0.6%, melted, maintained at 45°, and added volume:volume to dilutions of cell suspensions. Three ml of each cell suspension was plated in triplicate (3 wells/dilution of cell suspension) in Costar Cluster[6] plates (Costar, Cambridge, MA). Cells were incubated at 37° in a CO_2 incubator (5% CO_2, 95% air) for up to 7 weeks. Cells were fed at approximately 4 day intervals by adding 1–2 ml of semisolid growth medium to each well. An inverted microscope (10× lens) was used to score colonies at approximately 4 day intervals. A discrete cluster of cells was scored as a colony if it contained more than 50 cells (8).

Statistical methods. Statistical methods were described (9). Data from the dose-response assays for the SQ, SP, and IP lines were computer plotted by use of the Michigan Interactive Data Analysis System (MIDAS) which is a subroutine on the Michigan Terminal System. Tests included one-way covariance models for regression analysis, and Student's "t" test.

Results. Derivation of the SQ, SP and IP sublines. The starting material for all three subines was a SQ transplant (360-73) of WEHI-3B.

The SQ subline was maintained by serial passage at 3-week intervals. Tumors were harvested at their peak of growth and minced to 1 cu mm with sterile scalpels in cold (4°) MBSS to yield dispersed cell suspensions. After cell and tissue debris was allowed to settle (4–5 min at 4°) the cells remaining in suspension were washed twice. Viable cell counts were done by the trypan blue dye exclusion technique. Cells were suspended in MBSS to prepare 20% suspensions (v/v) and 0.5 ml was injected SQ between the scapulae of recipient mice. The *splenic (SP) subline* was derived from the SQ line by the ip injection of a 20% suspension of SQ tumor cells. Thereafter spleen cells from mice moribund from leukemia were used for serial passage by the ip route. Because the original sublines of WEHI employed by Warner, Moore and Metcalf (4) underwent significant variation on serial passage, the SP subline was passed serially 25× to adapt it fully to the splenic

growth form. Monodisperse spleen cell suspensions of the SP subline were washed twice in MBSS and viable cell counts done by the trypan blue dye exclusion technique. For routine passage 10^6 viable cells were injected ip. The *IP Subline* was derived from mice that had been inoculated with passage 27 of the SP subline. However, peritoneal cells obtained from mice moribund from leukemia were used for serial passage by the ip route instead of spleen cells. For routine passage 6.0 ml of MBSS were injected ip into mice moribund from AMML, the abdominal cavity gently massaged, and the fluid withdrawn with an 18 gauge needle and syringe. Cells were washed twice and counted. Viability consistently was 95–100%.

Morphologic studies. For morphologic studies Wright-stained films and hematoxylin–eosin stained sections of cell blocks were prepared from the cell suspensions used for routine passage. The morphologic properties of the cells in the SQ tumors were essentially the same as those described by Warner, Moore and Metcalf (4). Figure 1 shows the cell types characteristic of the SP subline as seen in cell block preparations. Many of the cells were large, atypical blasts, characterized by small amounts of cytoplasm and large, pleomorphic, round to irregular nuclei containing two to four nucleoli. Occasional dividing forms were present. Other large atypical cells were monocytoid with indented, lobulated, or folded nuclei or horseshoe-shaped, irregularly lobed, nuclei. Scattered amongst these elements were many lymphocytes, presumably of splenic origin, and occasional megakaryocytes and granulocytes. Cells of the IP subline were more homogeneous morphologically than the SP line because of the absence of admixed splenic elements.

Dose-response assays. Cell suspensions were prepared from each subline as described above. Viable cells in graded $\log_{10}$ dilutions were injected by the appropriate route into separate groups of mice. Mice were observed for 45 days after inoculation and deaths from leukemia were scored. Regression curves were computer analyzed as described previously (9). Data for the SQ subline were obtained for passages 8 and 10, from passages

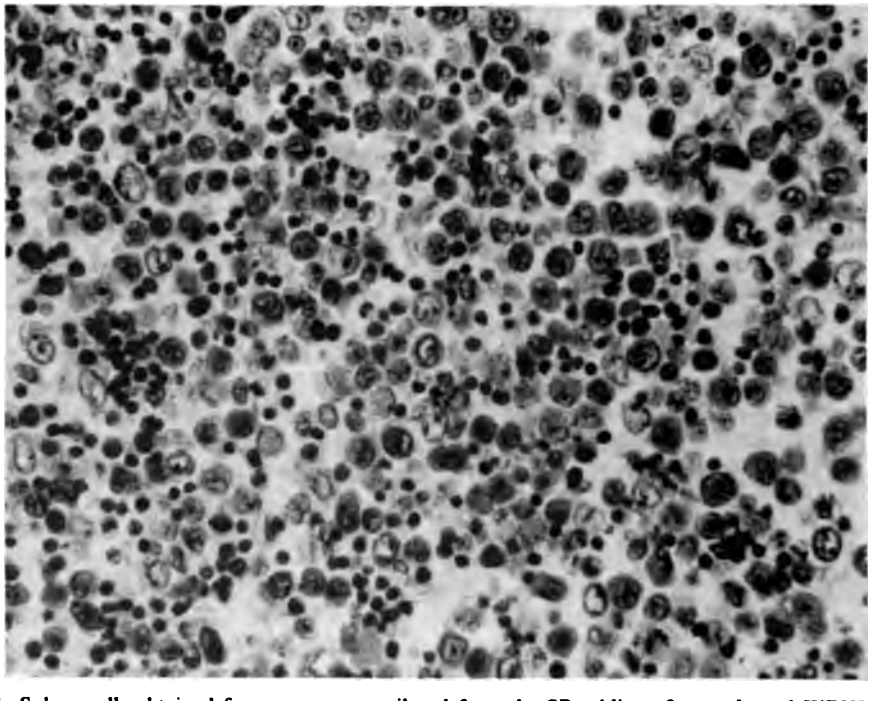
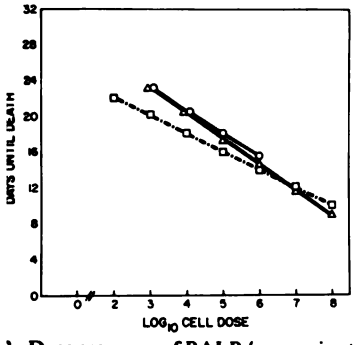


FIG. 1. Spleen cells obtained from a mouse moribund from the SP subline of transplanted WEHI-3B myelomonocytic leukemia were prepared as a cell block and sectioned. Large pleomorphic leukemic cells are mixed with lymphocytes. Magnification × 410; all sections were stained with hematoxylin–eosin.

nd 33, respectively for the SP subline, passages 1, 4, 6, and 20, respectively, P subline. The slopes of the regression for the serial passages of any single e (data not shown) did not differ sig- ly. When the data were pooled (Fig. s found that the three sublines did not ignificantly in their lethality for mice. any single experiment the standard ons in survival data were of the range .8. In practice, the spread in time in ntil death at any given cell dose with the three transplantation lines did not 1–5 days.

e cell kill for the IP subline. The sta- approach employed previously by us by Skipper, Schabel and Wilcox (6) ed to test whether a single tumor cell IP subline caused fatal AMML. A l discussion of the theoretical basis for lysis is presented by Skipper, Schabel lcox (6). In brief, the fraction of mice e from transplanted leukemia, when ceive an inoculum of two or less cells, ictable from the Poisson distribution here $P(m) = a^m \cdot e^{-a}/m!$ In this equa- m) is the fraction of mice that receive ible cells when the average number of cells injected per mouse is "a". Cell ions are diluted so that some mice 0 cells, some one cell, some two cells, ne can estimate the number of cells l into each mouse by applying the n distribution to evaluate $P(0)$, *i.e.,* ome mice survive because they receive



2. Dose response of BALB/cwm mice to graded es of the SQ (O· · · ·O), SP (Δ——Δ) and IP D) sublines of WEHI-3B myelomonocytic leu- ach point on each regression curve represents from pooled results (two to four experiments, ce per point).

0 cells. Under these assay conditions the equation simplifies to $P(0) = e^{-a}$. In our experiments we simply sought to determine whether the lethality of tumor cell suspensions was consistent with single cell kill. Thus, a suspension of IP cells (10^7 viable cells/ml) was diluted to contain (statistically speaking) one viable cell/ml of inoculum. In the experiment 68 mice received a 1 ml ip injection of the diluted preparation. Of these 7 died from transplanted AMML whence $P(0) = 61/68$ or $P(0) = 0.9$. Substituting 0.9 for $P(0)$ yields $0.9 = e^{-a}$ whence "a" becomes 0.11. Thus, under the test conditions the average number of leukemic cells injected per mouse was 0.11. The computed probability distribution is as follows: $P(0) = 61$ mice received no leukemic cells; $P(1) = 7$ mice received one leukemic cell; $P(2) = 0.005$ mice received 2 malignant cells. The actual experimental results were in excellent accord with the computed probability distribution. Thus, the findings provide evidence that a single malignant cell of the IP subline caused fatal AMML. Since the dose-response curves for all 3 sublines were so similar it is reasonable to assume that they all approximated single cell kill.

Hematologic studies. Detailed hematologic studies were done with the IP subline (passage 49). Each of 55 mice received a 1 ml injection of 10^4 viable cells ip. The experiment was terminated at 21 days because cumulative mortality reached 80–90% by this time and values were so variable in such moribund mice. The data summarized in Table I are based on the following definitions: Lymphocytes were assigned to a single category irrespective of size. Cells were scored as mature granulocytes if they had distinctly segmented nuclei and specific cytoplasmic granules. Granulocytes with slender, ringlike, but nonsegmented nuclei were classified as bands while those with heavier ringlike nuclei, or round to oval nuclei, were defined as metamyelocytes and myelocytes, respectively. These last three categories are classified under the single heading "immature granulocytes." Monocytes were identified by their abundant gray-blue cytoplasm and indented, lobulated, or folded nuclei. "Blast cells" were identified by their blue cytoplasm and large nuclei, a fine chromatin pattern, and usually multiple nucleoli. Some blast cells were only

slightly larger than the other circulating leukocytes while others were giant forms with a two to threefold larger volume. "Immature monocytes" were intermediate morphologically between blasts and monocytes.

Table I shows that hematocrits remained normal for 18 days but decreased approximately 20% during the last 2 to 3 days of life. Similarly, total leukocyte counts were essentially normal through day 17. Thereafter, elevation of total counts was pronounced with median values at days 18, 19, 20 and 21 of approximately 22,000, 28,000, 42,000 and 73,000 WBC/mm^3, respectively. This leukocytosis could be attributed largely to an increase in cells in the granulocyte compartment (mostly neutrophils, an occasional eosinophil, but no basophils). An increase in mature granulocytes was evident by 12 days after incubation with approximately a tenfold increase immediately before death. There was an fourfold increase in immature granulocytes by day 18 with a 16-fold increase before death. An increase in mature monocytes was evident by 18 days with approximately a tenfold increase before death. Immature monocytes and blast cells were detected as early as 8 to 16 days after inoculation and either remained elevated or increased in number up to death.

Histopathologic studies. 10^4 viable IP cells were injected ip and mice were killed at 4, 8, 12 and 16 day intervals. At 4 days malignant cells were not detectable in tissues. By 8 days there were neoplastic infiltrates in the intra-abdominal adipose tissue. At 12 days these infiltrates were larger and malignant cells were evident in the liver. Neoplastic cells were present in mediastinal and retroperitoneal lymph nodes but were not detected in the spleen or bone marrow. By 16 days infiltrates were more extensive and had begun to penetrate into the pelvic and abdominal viscera. Although the splenic red pulp was infiltrated by neoplastic cells, similar leukemic infiltrates could not be detected in the marrow. However, there were small, ill-defined foci in the marrow in which less mature forms seemed more numerous than is normal. Figures 3 and 4 show typical histopathologic changes. When mice were inoculated with the SP subline the results were essentially the same. When IP or SP cells were inoculated by the iv route no remarkable differences were found compared with the ip route.

Plating efficiency of cells. Although the three transplanted cell lines were essentially equal in their lethality for mice, the possibility existed that the proportions of tumor cells of different types comprising each line might differ as a result of selective pressures exerted during serial passage. The histologic studies described above indicated either qualitative or quantitative differences in the cell popu-

TABLE I. HEMATOLOGIC CHANGES IN MICE INOCULATED WITH THE IP SUBLINE OF WEHI-3B MYELOMONOCYTIC LEUKEMIA.

Days[a] after inoculation	Cells per cu mm × 10^{-3}							
	Hematocrit	Total WBC	Lymphocytes	Granulocytes[b]	Immature Granulocytes[c]	Monocytes	Immature monocytes	Blasts[d]
4	55.6 ± 2.0	11.3 ± 2.4	9.1 ± 2.3	1.7 ± 0.3	0.1 ± .1	0.2 ± 0.1	0.0 ± 0.0	0 ± 0
8	56.4 ± 5.7	13.2 ± 3.6	10.3 ± 3.3	2.4 ± 0.8	0.2 ± .3	0.3 ± 0.3	0.0 ± 0.0	.1 ± .1
12	57.1 ± 9.8	17.6 ± 3.7	12.6 ± 4.3	3.9 ± 2.5	0.2 ± .2	0.2 ± 0.3	0.0 ± 0.1	.1 ± .2
16	50.9 ± 3.1	13.8 ± 2.9	7.4 ± 2.6	5.1 ± 1.4	0.3 ± .3	0.4 ± 0.2	0.2 ± 0.2	.4 ± .2
17	53.4 ± 1.9	13.6 ± 3.0	6.9 ± 1.7	5.6 ± 2.1	0.2 ± .2	0.2 ± 0.2	0.2 ± 0.1	.3 ± .2
18	51.7 ± 5.5	21.8 ± 7.8	10.0 ± 5.5	8.3 ± 4.0	0.4 ± .1	0.7 ± 0.4	0.7 ± 0.5	.4 ± .3
19	47.3 ± 3.8	28.5 ± 13.1	11.5 ± 7.0	14.4 ± 5.7	0.8 ± .9	1.4 ± 1.5	0.1 ± 0.2	.2 ± .3
20	43.9 ± 5.4	41.7 ± 14.6	17.6 ± 7.9	19.2 ± 5.8	1.0 ± .8	2.2 ± 1.1	0.4 ± 0.5	1.2 ± 1.5
21	41.6 ± 3.3	72.8 ± 46.0	35.0 ± 20.0	30.0 ± 23.8	1.6 ± 1.7	3.9 ± 2.8	0.5 ± 0.7	1.8 ± 2.0
18[e]	54.1 ± 2.1	14.3 ± 3.3	11.1 ± 3.1	2.7 ± 0.7	0.1 ± .2	0.4 ± 0.3	0.0 ± 0.0	0 ± 0

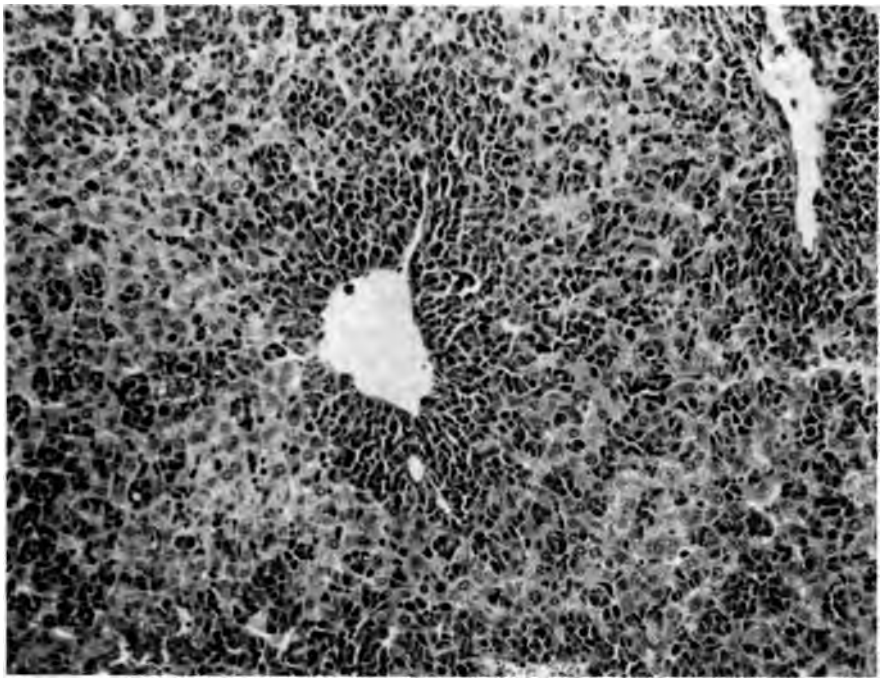
[a] 10^4 viable IP cells were injected ip into separate groups of mice so that most animals were bled only once. Each data point represents the results from 7 animals.

[b] Polymorphonuclear neutrophils and eosinophils.

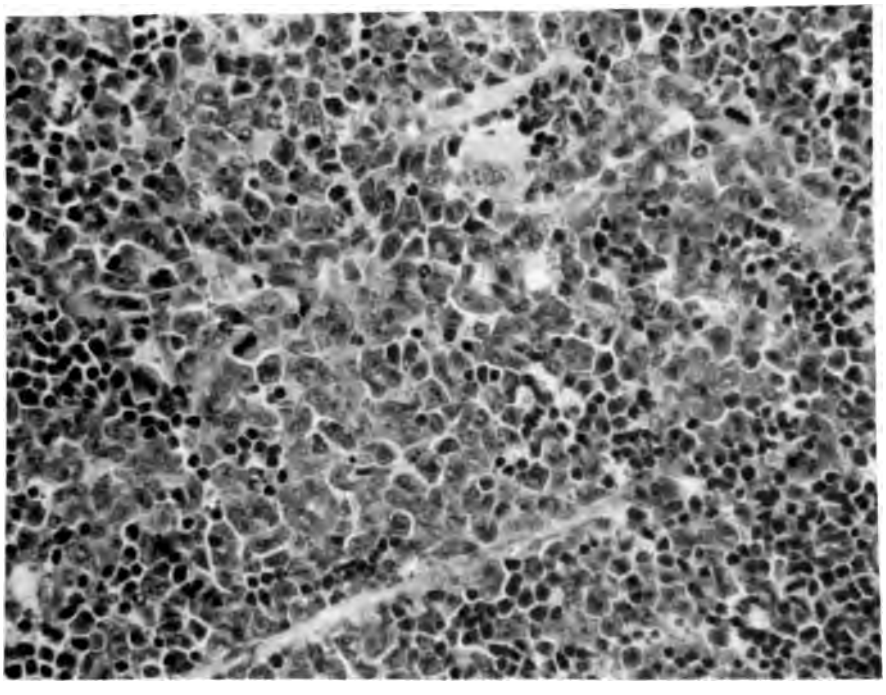
[c] Myelocytes, metamyelocytes, and bands.

[d] Undifferentiated stem cells.

[e] Control mice inoculated with MBSS, and killed either on the day of injection or 18 days later. Since counts were essentially the same for both sets of mice, data are presented only for the 18 day animals.



IG. 3. Liver from a mouse moribund from the IP subline of transplanted myelomonocytic leukemia. The itic parenchyma is partly replaced by deeply staining leukemic cells that are particularly dense perivascularly 60).



IG. 4. Spleen from a mouse moribund from the IP subline. The red pulp is largely replaced by pleomorphic ocytoid cells that contrast with the smaller darker lymphocytes (× 410).

lations in the three cell lines. In an attempt to obtain more objective data we compared the plating efficiency of the SQ, SP, and IP sublines. Freshly harvested cells of each tumor line were diluted serially, plated in semisolid medium, and incubated at 37° for up to 7 weeks. Cell growth was scored by examining plates at approximately 4 days intervals. Clusters of 50 or more cells were scored as colonies. "Compact" colonies were those that grew rapidly (were detectable macroscopically in 10–15 days and were 1 to 2 mm in diameter), had very dense centers, and were surrounded by a narrow border of cells growing into the agar. "Diffuse" colonies usually grew more slowly, did not have compact centers, and cells were diffusely distributed in the semisolid agar. No attempt was made to identify the cell type in colonies as was done by Metcalf, *et al.* (8). Even though the data in Table II are somewhat of a preliminary nature they reveal rather clear cut differences in the growth capabilities of cells of the SQ, SP, and IP lines. Colonies failed to grow out from the SQ and SP lines if the inoculum contained less than 10^5 cells per ml. At the 10^5 inoculum growth was so dense that colonies overlapped and accurate counts could not be made. A rather wide range of colony types was observed with the SQ line. With the SQ line colonies could not be detected macroscopically until cultures were 4–5 weeks old. The SP line showed the same threshold effect as the SQ line, although colonies were usually of the compact or diffuse type. Colonies were detectable macroscopically after 10–15 days of incubation. When a cell inoculum of 3×10^2 or 3×10^1 of the IP line was used, plating efficiency was of the order of 5%, colonies became visible macroscopically in 10–15 days, growth was essentially the same in medium containing a final concentration of either 10 or 20% fetal calf serum, colonies were either of the diffuse or compact type, and the two colony types occurred in equal proportions.

Discussion. To develop a practical model of transplantable AMML in mice three sublines of the WEHI-3B tumor were adapted by serial passage in syngeneic BALB/c mice. The SQ subline was employed as a model to simulate solid tumor growth, the IP subline to represent the ascitic growth form, and the SP subline as a model for splenic transmission. For each subline the time in days until death was a linear function of the $\log_{10}$ cell dose inoculated (Fig. 2). The slopes of the regression curves of the three sublines were essentially the same and remained stable after each subline was fully adapted by serial passage. The stability of the cell lines demonstrated in this report was important because Warner, Moore and Metcalf (4) described marked changes among subpassages of WEHI cells on serial transplantation. Variability in the properties of transplantable lines of rat AMML has been a problem in the development of some of these models (10).

The main value of the dose-response data is that they permit precise quantification of AMML tumor cell populations. Thus, it becomes possible by use of techniques described previously (11) to quantify how immunologic reactions or drugs affect the course of the experimental disease. However, the morphologic diversity of AMML cell populations was

TABLE II. PLATING EFFICIENCY OF THE SQ, SP, AND IP SUBLINES OF WEHI-3B.

Cell line	Passage numbers	% plating efficiency with inoculum concentrations:[a] 10^5	10^4	10^3	10^2	10^1	Time for colonies to become visible macroscopically	Colony types[c]
SQ	12	TNC[b]	0	0	0	0	4 to 5 weeks	Diverse
SP	37	TNC	0	0	0	0	10 to 15 days	Diffuse or compact
IP	103, 107	TNC	TNC	TNC	4.97	4.95	10 to 15 days	Diffuse or compact

[a] Percentage of viable cells inoculated into plates that developed into discrete colonies containing 50 or more cells each. Cells at the indicated densities were inoculated into Costar Cluster[6] plates using triplicate wells/cell dilution. Cells were incubated at 37° for up to 7 weeks in a CO_2 incubator. Cells in plates were fed at approximately 4 day intervals by adding 1–2 ml of fresh semisolid growth medium. Plating efficiencies were calculated by dividing the number of colonies/plate by the number of viable cells in the inoculum × 100. Results are from two representative experiments.

[b] Colonies were Too Numerous to Count.

[c] See text for detailed description.

plicating factor, i.e., the slopes of the ıion curves may have represented the ffect of interacting cell populations. it was important to determine if the ty of a single malignant myelomono- ɛukemic cell was altered by other cells ɔd in the same test population. The of the statistical analysis with the IP ɛ provided evidence consistent with sin- l kill, i.e., the presence of nonmalignant ı preparations did not affect the malig- of single IP cells under these test con- ɔ.

ıough the dose-response and histopath- data indicated that all three sublines similar, they could be distinguished ɔach other by the use of more sensitive techniques. For example, the data in II shows that the plating efficiency of line was approximately 5%, colonies m in size grew out in 10–15 days, and ɛither compact or diffuse. In contrast, ' and SQ lines both showed a marked hold effect", i.e., colonies failed to grow ıen an inoculum of $<10^5$ cells was used. ɛ 10^5 concentration colonies grew at ıgh densities that they overlapped each For the SP line colonies grew out to scopically visible size in 10–15 days ere either "compact" or "diffuse." For) line colonies were not detectable mac- ıically until 4–5 weeks of incubation rather diverse array of colony types ɔbserved. While the data in Table II are ɛdly of a preliminary nature, they heless provide rather convincing evi- for qualitative differences in the cell ıtions that comprise the three trans- ble lines. Diversity within a given line as been documented. For example, 13 l lines of cells of been established in ɛ from the IP line. They differ in their ıologic properties, growth characteris- ıd relative malignancy for BALB mice. ɪample, the LD_{100} dose of a line desig- A3 is 10^6 viable cells with a mean al time of mice of 50.3 days. For a line ɹated MW-7, the LD_{100} dose is $<10^3$ and survival time is 32.2 days. Detailed ɜ of the type described by Metcalf *et al.* ɛ being carried out to more adequately ɪterize the cell populations that com- he SQ, SP, and IP lines.

The histopathologic changes caused by the IP subline were consistent with the initial multiplication of the malignant cells in various abdominal organs and tissues. After progressive cell growth and infiltration of tissues, cell populations reached proportions sufficient to appear in the peripheral blood. Such results are typical of transplanted leukemias. In the original report by Warner, Moore and Metcalf (4) detailed hematologic data were not provided for the WEHI-3B line. Because of the recognized difficulties in the precise identification of the cell types that are found in AMML (12) we classified cells into major categories that could be identified with reasonable accuracy. The most impressive hematologic change was the increase in the cell populations in the granulocyte compartment (neutrophils). An increase in mature granulocytes occurred as early as 12 days after inoculation and became approximately tenfold before death. A similar pattern occurred with the cells classified as "immature" granulocytes (principally metamyelocytes and bands). Blast cells were detected in the peripheral blood as early as 8 days after inoculation and increased in number until death. Mature and immature monocytes increased in the peripheral blood by 18 days after inoculation and remained elevated until death. These hematologic changes, and the accompanying pathologic changes in tissue, were consistent with characterization of the disease as myelomonocytic leukemia as reported originally (4). We also confirmed that invasion of bone marrow was minimal in the transplanted disease. This does not necessarily detract from the experimental model since it will be of interest to determine whether the failure of WEHI-3B cells to infiltrate the bone marrow in large numbers is immunologically mediated or has some other basis. For example, resistance to allografts of bone marrow in mice appears to be mediated (13) by a ^{89}Sr-sensitive bone marrow effector cell (M cells) and is relatively independent of classical B and T cell immunologic mechanisms.

The proliferative changes observed in tissues, as well as some of the hematologic alterations in the peripheral blood, are similar to those found in other transplanted myeloid leukemias. For example, in their studies of RMF transplanted leukemia Husseini *et al.*

(14) also found a stimulatory effect of transplanted myeloid leukemia cells on normal hematopoiesis. Studies in progress have shown that a cloned line of WEHI-3B cells (HUCL-1) produces colony stimulating factor (CSF) *in vitro*. Perhaps the production of CSF *in vivo* by malignant WEHI cells accounts for some of the hematopoietic or histopathologic changes seen in this disease. It also may account for the "threshold effect" observed with the SQ and SP lines in the plating efficiency tests. Thus, it will be of interest to compare various cloned lines of WEHI-3B cells for their relative malignancy *in vivo*, their capacity to produce CSF, and accompanying histopathologic changes. The current study was done to carefully define the disease so that the results of such experiments could be evaluated accurately.

Summary. Three transplantable sublines of WEHI-3B myelomonocytic leukemia in BALB/c mice were developed by splenic (SP), intraperitoneal (IP) and subcutaneous (SQ) passage. All three sublines then were compared for stability on passage, lethality for mice, and major hematologic and histopathologic effects. The time in days until death was a linear function of the $\log_{10}$ dose of viable cells injected for each cell line. Evidence was provided that a single cell of the IP line was lethal to mice and that death from all three cell lines approximated single cell kill. All three sublines were stable after adaptation by serial passage. Detailed hematologic and histopathologic studies were done on mice that were inoculated iv and ip with the SP and IP sublines. Changes typical of myelomonocytic leukemia were observed except that bone marrow invasion was minimal.

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Effects of Synthetic Vasoactive Intestinal Peptide (VIP), Secretin and Their Partial Sequences on Gastric Secretion[1] (40097)

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VIP, secretin, glucagon and GIP (Gastric Inhibitory Peptide), in this order, constitute a series of peptides characterized by increasing structural homology with GIP (1). The first pair, VIP and secretin, exhibit considerable functional homology: they share effects on fat (2), liver (3), exocrine pancreas (4), and colonic mucosa (5) which appear to be mediated by the same set of receptor sites. Glucagon and GIP share few effects with VIP and secretin; even where the effects are shared they appear to be mediated by separate sets of receptor sites or intermediary mechanisms (6, 7). All four peptides inhibit gastric acid secretion (1) but have different effects on pepsin secretion; the mechanism(s) of inhibition remain unknown. Barbezat and Grossman (8) were first to report that natural porcine VIP inhibited histamine-stimulated acid secretion in dog Heidenhain pouches.

In the present study, we show that synthetic porcine VIP inhibits pentagastrin-stimulated acid and pepsin secretion in the dog. The inhibition differs in some respects from that produced by synthetic porcine secretin. Since these results were first reported (9), Konturek *et al.* (10) have shown that natural VIP inhibits histamine-stimulated acid and pepsin secretion in the dog and Villar *et al.* (11) have shown that natural VIP inhibits food-stimulated acid as well as gastrin secretion.

Materials and methods. Gastric secretion was measured in three dogs (15–20 kg body weight) equipped with gastric fistulae. Following an overnight fast, basal secretion was collected for a period of 30 min. Pentagastrin was then infused in a dose of 0.25, 1 or 4 $\mu g\ kg^{-1}\ hr^{-1}$ for 3 hr. Sixty to seventy-five min after the start of pentagastrin infusion, synthetic porcine VIP (0.5 $\mu g\ kg^{-1}\ min^{-1}$) or synthetic porcine secretin (0.05 $\mu g\ kg^{-1}\ min^{-1}$) was infused for 7.5 min. VIP was given once and secretin twice to each dog with each dose of pentagastrin. In control studies, pentagastrin was given alone without VIP or secretin (nine experiments).

The effect of carboxyl-terminal partial sequences of VIP (VIP_{14-28}, VIP_{15-28} and VIP_{18-28}) was examined on a background of 1 $\mu g\ kg^{-1}\ hr^{-1}$ of pentagastrin. The three fragments were infused sequentially for 7.5 min at 45-min intervals one hour after the start of pentagastrin infusion (three experiments). The dose of each was 1.8 $\mu g\ kg^{-1}\ min^{-1}$ corresponding to about 8 times the molar dose of VIP used in this study.

The effect of carboxyl-terminal partial sequences of secretin was also examined on a background of 1 $\mu g\ kg^{-1}\ hr^{-1}$ of pentagastrin. All secretin fragments were given once per session one hour after the start of pentagastrin infusion. $Secretin_{5-27}$ was given twice to each dog as a prompt intravenous injection of 5 $\mu g\ kg^{-1}$. A substituted analogue of $secretin_{5-27}$ in which the glutamic acid in position 9 is substituted by glutamine (9-Gln-$secretin_{5-27}$) was infused twice in each dog in a dose of 0.5 $\mu g\ kg^{-1}\ min^{-1}$ for 7.5 min. Finally, 9-Gln-$secretin_{5-27}$ was infused twice in each dog in a dose of 0.5 $\mu g\ kg^{-1}\ min^{-1}$ in combination with secretin 0.05 $\mu g\ kg^{-1}\ min^{-1}$ for 7.5 minutes. The doses of secretin fragments were about 12 times the molar dose of secretin used in this study.

Secretion was collected at 15-minute intervals throughout the experiments. Acid concentration was measured by electrometric titration to pH 7.5 and pepsin was measured by the method of Hunt (12) and expressed in mg of pepsin standard.

Synthetic porcine VIP and partial sequences of VIP and secretin were kind gifts of Dr. Miklos Bodanszky, Department of

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Chemistry, Case Western Reserve University. Synthetic porcine secretin was a kind gift of Dr. Miguel Ondetti, Squibb Institute for Medical Research, New Jersey.

Results. Effect of synthetic VIP and partial sequences of VIP. Infusion of synthetic VIP in a dose of 0.5 $\mu g\ kg^{-1}\ min^{-1}$ for 7.5 minutes produced a prompt inhibition of acid secretion which reached a nadir in the first 30 min (Fig. 1). The extent of inhibition was determined by comparing the rate of acid secretion in the 30-min periods before and after the start of infusion of VIP (Fig. 2). Inhibition at the lowest dose of pentagastrin (57 ± 7%) was significantly greater than inhibition at the highest dose (23 ± 7%) ($P < 0.01$). Dose-response analysis (response versus response/dose) indicated that inhibition was of the competitive type: the maximal acid responses for the period before (15.6 ± 0.8 meq/30 min) and after VIP (13.8 ± 2.2 meq/30 min) were not significantly different but the D_{50} for pentagastrin increased twofold from 0.47 ± 0.05 to 1.02 ± 0.22 $\mu g\ kg^{-1}\ hr^{-1}$ ($P < 0.05$) in the presence of VIP. An apparent inhibitor dissociation constant for VIP was calculated to be 0.43 $\mu g\ kg^{-1}\ min^{-1}$.

VIP inhibited also the maximal pepsin response by 65 ± 10% ($P < 0.01$) but it had no effect on the reversed pepsin response observed at higher doses of pentagastrin (Fig. 2).

None of the carboxyl-terminal partial sequences of VIP had any detectable effect on pentagastrin-stimulated acid or pepsin secretion.

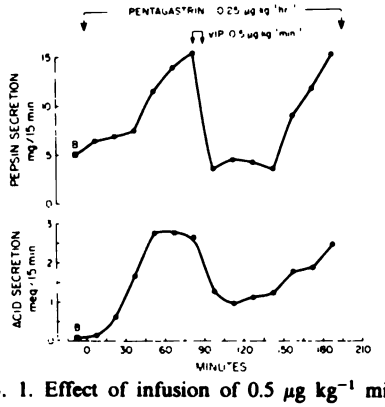
PENTAGASTRIN 0.25 µg kg⁻¹ hr⁻¹
VIP 0.5 µg kg⁻¹ min⁻¹
PEPSIN SECRETION mg/15 min
ACID SECRETION meq/15 min
B
0 30 60 90 120 150 180 210
MINUTES

FIG. 1. Effect of infusion of 0.5 $\mu g\ kg^{-1}\ min^{-1}$ of synthetic porcine VIP for 7.5 min on pentagastrin-stimulated acid and pepsin secretion (mean of three experiments). B = basal secretion.

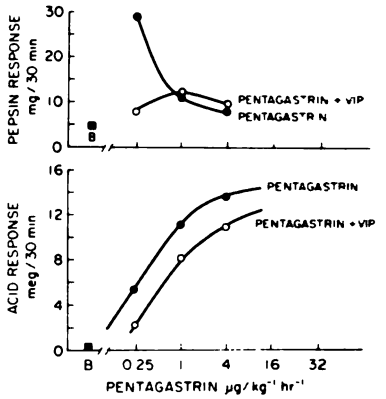

FIG. 2. Dose-response curves for the effect of pentagastrin infusion alone and in combination with VIP (0.5 $\mu g\ kg^{-1}\ min^{-1}$ for 7.5 minutes) on acid and pepsin secretion. Each point is the mean of three experiments. The highest pepsin response was attained at the lowest dose of pentagastrin (0.25 $\mu g\ kg^{-1}\ hr^{-1}$). B = basal response.

Effect of synthetic secretin and partial sequences of secretin. Infusion of synthetic secretin in a dose of 0.05 $\mu g\ kg^{-1}\ min^{-1}$ also produced a prompt inhibition of acid secretion which reached a nadir in the first 30 min (Fig. 3). Percent inhibition was the same at all dose levels of pentagastrin: 66 ± 3%, 60 ± 4% and 60 ± 8% at 0.25, 1 and 4 $\mu g\ kg^{-1}\ hr^{-1}$ of pentagastrin respectively. Accordingly, a linear relationship characteristic of inhibition of the non-competitive type prevailed between the responses before and after secretin (Fig. 4). From the slope in Fig. 4, an apparent inhibitor dissociation constant for secretin was calculated to be 0.03 $\mu g\ kg^{-1}\ min^{-1}$.

Neither secretin$_{5\text{-}27}$ given by prompt intravenous injection nor 9-Gln-secretin$_{5\text{-}27}$ given by intravenous infusion had any detectable effect on pentagastrin-stimulated acid secretion (Fig. 3). 9-Gln-secretin$_{5\text{-}27}$ infused in a dose twelve times the dose of secretin failed to block the inhibitory effect of secretin.

Discussion. VIP and secretin share a number of secretory (10), metabolic (13), motor (14) and hormone-releasing (13) or inhibiting (11) properties. These are thought to reflect similarities in the structure of the two peptides which in turn determine binding to common regulatory sites and activation of common intracellular effector (messenger) systems. Inhibition of gastric acid secretion in

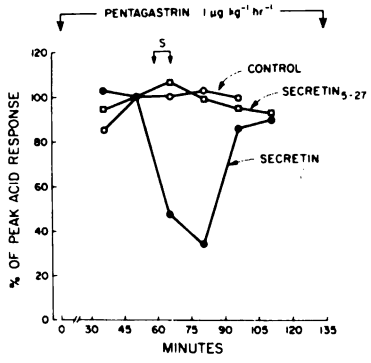

FIG. 3. Effect of infusion of 0.05 $\mu g\ kg^{-1}\ min^{-1}$ of synthetic porcine secretin (closed circles) or 0.5 $\mu g\ kg^{-1}\ min^{-1}$ of 9-Gln-secretin$_{5-27}$ (open squares) for 7.5 min on pentagastrin-stimulated acid secretion. Control secretion (open circles). Each point is the mean of six experiments. Responses expressed in percent of the response immediately preceding (i.e. 45–60 min) the infusion (S) of secretin of its analogue.

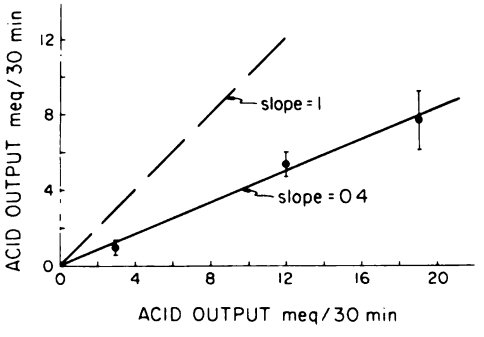

FIG. 4. Linear relation between the response to pentagastrin alone (horizontal axis) and the corresponding response to pentagastrin plus secretin (vertical axis). Each point is the mean of six experiments. A slope of 0.4 indicates 60% inhibition at all doses of pentagastrin. Slope = $1/(1 + i/K_i)$ where i = dose of secretin and K_i = inhibitor dissociation constant.

the dog appears to conform in some respects to this pattern; however, several differences emerge. First, as shown here for synthetic porcine VIP and secretin and elsewhere for the natural forms (8, 10, 11), both peptides inhibit stimulated acid secretion in the innervated stomach but only VIP inhibits histamine-stimulated acid secretion in a denervated pouch (8). Secondly, inhibition of acid secretion by VIP appears to be of the competitive type, whereas inhibition by secretin is of the noncompetitive type (15) (Fig. 4). Thirdly, inhibition by VIP extends to histamine- and pentagastrin-stimulated pepsin secretion.

In the present study, synthetic VIP appeared to be less effective than synthetic secretin, at least in terms of the doses required for an equivalent degree of inhibition. The difference is unlikely to be due to an artefact in the synthesis of either peptide. Konturek *et al.* (10) found a similar difference in potency between the natural forms of VIP and secretin. Furthermore, the inhibitor dissociation constant for synthetic VIP (0.4 $\mu g\ kg^{-1}\ min^{-1}$) was similar to that reported by Barbezat and Grossman (8) for natural VIP. Finally, the batch of synthetic VIP used in this study was tested against several target tissues and found to possess the spectrum of properties displayed by natural VIP (13). In particular, it was shown to be an effective intestinal secretory stimulant in the dog equipotent in this respect to natural porcine VIP (13).

A low potency in vivo precludes a role for VIP as an endocrine inhibitor of gastric acid secretion. The presence of VIP-secreting cells or nerves in the fundus and the antrum of the mammalian stomach (16, 17) argues in favor of a paracrine or neurocrine role for VIP in the control of gastric secretion, either directly by inhibition of gastric acid and pepsin secretion or indirectly by inhibition of antral gastrin secretion.

The decrease in gastric secretion which is a common feature of the "watery diarrhea" syndrome has been attributed to high levels of circulating VIP (18, 19). This attribution remains to be validated by simultaneous immunometric and secretory measurements and by the exclusion of other causes, such as dehydration and hypokalemia, for the decrease in gastric acid secretion.

The synthetic partial sequences of VIP and secretin used in this study are known to be active on or to retain binding to some tissues such as the exocrine pancreas (20) and the colonic mucosa (5) in some species. However, at the relatively small doses used, none showed an inhibitory effect on gastric acid secretion.

Summary. The effects of synthetic porcine VIP and secretin on pentagastrin-stimulated gastric secretion were compared in dogs equipped with gastric fistulae. VIP inhibited both acid and pepsin secretion. Percent inhi-

bition of acid secretion by VIP varied with the dose of pentagastrin: dose-response analysis showed inhibition to be of the competitive type. Percent inhibition of acid secretion by secretin did not vary with the dose of pentagastrin and was consistent with inhibition of the noncompetitive type. Carboxyl-terminal partial sequences of VIP and secretin, at approximately 10 times the molar doses of the parent peptides, had no detectable inhibitory effect.

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Effect of Heparin on Cytomegalovirus Replication[1] (40098)

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eral studies have shown that heparin its the infectivity of many members of erpesvirus group. Inhibition of herpes ex virus (HSV) infectivity was shown to ise as the concentration of heparin was ised (1, 2). It was also reported that in sensitivity can be of value in distining HSV-1 from HSV-2 strains since type 2 strains were found to be insensi- treatment with this drug (3, 4). Other sviruses including pseudorabies virus, lla virus and human cytomegalovirus were also found to be inhibited by this ally occurring biological compound.

ring the course of studies on the develnt of guinea pig cytomegalovirus MV) viremia, it was noted that loss of MV infectivity occurred in the presence parin. Analyses of the effect of heparin e replication of GPCMV, together with minary studies on human, simian and ie CMV are included in this report.

terials and methods. Virus stock. The type guinea pig cytomegalovirus MV), strain 21222, originally isolated artley *et al.* (7) was obtained from the ican Type Culture Collection. Guinea erpeslike virus (GPHLV) strain LK40, olated in our laboratory (8). The human strain AD169 and the green monkey strain CSG, were obtained from Dr. ice Rowe, NIH, Bethesda, MD. The strain of mouse CMV was obtained Dr. John Booss, VA Hospital, West n, CT.

sue culture and virus assay. Primary a pig embryo (GPE) cell cultures were ared from 20- to 40 day-old embryos and grown in Eagle's medium supplemented with 10% calf serum as previously described (9). Passaged GPE cell cultures were used for assaying the guinea pig virus. The human WI-38 cell line was used for assaying both human and monkey CMV and mouse embryo fibroblasts (ME) were used for mouse CMV assay. Laboratory grade sodium heparin powder was purchased from Fisher Scientific Company. A stock solution was prepared in 0.9% saline (1 U = 10 μg). For virus assay, both virus induced cytopathic effect (CPE) and virus induced intranuclear inclusions in hematoxylin-eosin stained preparations were used to determine end points.

Results. Effect of heparin on cytomegalovirus replication. The degree to which the presence of heparin in the culture medium affected replication of four different CMV types is summarized in Table I. Growth of all of the CMV types tested was inhibited in the presence of a concentration of heparin comparable to that used for blood collection. The guinea pig and monkey CMV types showed the greatest reduction in infectivity when subjected to this treatment.

Comparison of inhibitory effect of heparin on GPCMV and GPHLV. Two herpesviruses of guinea pigs, GPCMV and GPHLV, were tested in cell culture in both the presence and absence of heparin. Virus suspensions containing 100 $TCID_{50}$ were inoculated into cultures containing different concentrations of heparin. The results are summarized in Table II. The presence of as little as 0.1 U/ml of heparin resulted in a tenfold reduction in the infectivity titers of GPCMV. Increased concentrations of heparin resulted in increased losses in infectivity accompanied by a corresponding delay in appearance of CPE, demonstrating a close relationship between the virus concentration and the inhibitory action of heparin. However, complete inhibition could not be obtained even with 100 U of heparin. In contrast, virus infectivity titers

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whom requests for reprints should be addressed.

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TABLE I. EFFECT OF HEPARIN ON REPLICATION OF SELECTED CYTOMEGALOVIRUS STRAINS IN CELL CULTURE.

Cytomegalovirus tested	Virus strain	Cell culture system	Average virus infectivity titers log $TCID_{50}$/ml		
			In heparin 100 U/ml	In absence of heparin	Log reduction
Human	AD 169	WI-38[a]	2.0	3.5	1.5
Green monkey	CSG	WI-38	1.5	5.0	3.5
Guinea pig	21222	GPE[b]	2.0	5.5	3.5
Mouse	Smith	ME[c]	2.0	3.5	1.5

[a] WI-38: Human fibroblast cell strain.
[b] GPE: Primary guinea pig embryo fibroblast.
[c] ME: Primary mouse embryo fibroblast.

TABLE II. COMPARISON OF HEPARIN EFFECT ON TWO HERPESVIRUSES OF GUINEA PIGS.

Concentration heparin (unit/ml)	GPCMV		GPHLV	
	Virus titer Log $TCID_{50}$/ml	Reduction (log)	Virus titer log $TCID_{50}$/ml	Reduction (log)
0	5.5	—	4.0	—
0.1	4.5	1.0	4.5	0
1.0	4.5	1.0	3.7	0.3
10.0	3.5	2.0	3.8	0.2
100.0	2.0	3.5	4.0	0

were not significantly reduced when GPHLV was treated in a similar manner.

Effect of heparin before or after virus adsorption. In order to understand the mechanism by which GPCMV replication was inhibited by the heparin, GPE cells on coverslips were treated with various concentrations of heparin for 1 hr at 35° before inoculation of 100 $TCID_{50}$ of virus (Table III, Expt. A). When cultures without heparin showed visible CPE, all inoculated cultures were fixed, stained and examined for the number of infected versus noninfected cells, expressed as percent reduction in comparison with cultures without heparin. Again, a reduction of approximately 80% in the number of GPCMV induced inclusions was observed when 1 U of heparin was added to cultures before virus adsorption and reached nearly 100% at higher concentrations. In experiment B, cultures were treated as in experiment A except that medium containing various concentrations of heparin was added to cultures one hour after virus adsorption. Inclusion formation was reduced by 50% at 1 U/ml of heparin and at 100 U/ml, a 68% reduction was obtained. However, in a separate experiment, when the adsorption period was extended to 3½ hr before addition of medium containing heparin, there was no significant difference in number of GPCMV-induced inclusions in cultures with or without heparin.

Comparison of different blood anticoagulants on the replication of CMV. The infectivity titers of GPCMV and monkey CMV when grown in cell culture medium containing different anticoagulants, were studied. GPCMV infectivity was significantly reduced in the presence of heparin, but no effect was observed when Alsever's solution, sodium citrate solution (3.8% in saline), or ethylene–diamine–tetra acetic acid (EDTA) were tested in a similar manner. Essentially the same results were obtained with the monkey CMV strain, in that only heparin reduced the infectivity when it was present in the cell culture medium.

Discussion. Sensitivity to heparin, as measured by decreased infectivity, has been shown to be characteristic of a number of different viruses and in particular appears to be a property of certain members of the herpesvirus group (5, 6). Inhibition of virus replication by heparin has been used as a basis for differentiating HSV types 1 and 2, when used in conjunction with other biological characteristics (3, 4). In the present study, the heparin sensitivity of GPCMV but not GPHLV, provides a useful tool for differentiating between these two endogenous herpesviruses of guinea pigs, thus enabling an investigator to inhibit GPCMV replication in those instances when a mixed infection with both herpesviruses was present in the same animal.

In addition, data was obtained confirming previously published reports (5), showing that

TABLE III. EFFECT OF HEPARIN TREATMENT BEFORE OR AFTER GPCMV ADSORPTION.[a]

ıt.	Method of assay	Heparin unit/ml	No. of inclusions per 1000 cells	Percent reduction
	Heparin added before virus adsorption	0	981	—
		0.1	560	43
		1.0	201	80
		10.0	1	99.9
		100.0	0.2	99.9
	Heparin added one hour after virus adsorption	0	406	—
		0.1	164	59.7
		1.0	201	50.5
		10.0	142	65.1
		100.0	128	68.5

ılum 100 $TCID_{50}$.

ıbitory process occurred prior to virus nent and penetration and that the hep- erted no influence on virus replication nfected cells were exposed to the com-

ırlier studies conducted in our labora- ɔn the pathogenesis of different her- ıses in experimental animals, heparin ed as an anticoagulant for blood col- in studies of viremia. No discernible lty in recovering either GPHLV (9) or HLV (10) was observed. However, btained in the present report indicate ; degrees of sensitivity to heparin by IV as well as the monkey, mouse and CMV types. For this reason the hep- nsitivity of these virus types should be into account when studies of CMV ı are carried out in these species. The :galoviruses tested did not appear to be 'e to any of the other commonly used ıgulants including Alsever's solution, ı citrate and EDTA. Furthermore, the :ported here show that the cytomega- es isolated from the mouse, guinea pig onkey should be added to the list of :galovirus types previously reported to ıitive to heparin.

mary. The presence of heparin in cell medium was found to reduce the infectivity titers of cytomegaloviruses isolated from human, monkey, guinea pig and mouse. This inhibition was shown to increase as the heparin concentration was increased. When heparin was added to cultures after virus adsorption had taken place, the effect on virus infectivity was negligible. Other anticoagulants studied included Alsever's solution, sodium citrate and EDTA, none of which showed any inhibitory effect on cytomegalovirus replication in cell culture.

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The Influence of Rat Age on Crypt Cell Kinetics after Partial Resection of the Small Intestine[1] (40099)

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Any investigator assumes *a priori* that data derived from studies utilizing different ages of animals cannot be rigorously compared. Several papers dealing with the compensatory response to intestinal resection have utilized animals of quite different age and weight and rarely has the stock been the same (see Table I). This study was designed to test the premise that the number of crypts per small intestine varies with animal weight and that indigenous crypt number will influence some features of the compensatory response of the crypt and villus cell population normally seen after intestinal resection (1-3).

A reduction in the total villus and crypt cell population by surgical removal of a portion of the intestine is soon followed by compensatory responses, the most prominent being alteration in values for several parameters of mucosal cell kinetics (2, 3).

The compensatory response to partial resection was assessed by the determination of (a) proliferative activity per crypt, (b) proliferative activity per milligram of tissue, (c) the number of crypts per milligram of tissue, and (d) the amount of tritiated thymidine (^{3}HTdR) incorporated per epithelial cell nucleus. The values obtained from the above measurements were compared with values obtained from control animals and those with varying degrees of intestinal resection.

Materials and Methods. Male juvenile (70 ± 10 g) and adult (350 ± 30 g) rats of the Holtzman and Sprague Dawley stocks were used. Two stocks of animals were used because not enough animals of one stock could be obtained at the time of the experiment.

The animals underwent transection, resection of the small intestine, or a simple laparotomy. The procedures were the same as described elsewhere (2). The mid-portion of the combined ileum and jejunum was transected or resected as noted below and in Table II.

Four groups, i.e., adults and juveniles of both stocks, were studied. In each of the four groups, 5° of resection were performed (0, 10, 50, or 80% resection or simple transection) with five animals per category.

The animals were housed in a room with constant temperature and a 12-hr light-dark cycle in wire bottom cages in groups of 6-8. For the first 24 hr postoperative, the animals were allowed 5% sucrose. Ten grams of laboratory chow per animal were allowed during the second 24-hr postoperative period. Laboratory chow and tap water *ad libitum* were available beginning the third postoperative day.

Fourteen days after a surgical procedure, [which is 2 days after the compensatory response reaches a plateau (1)], the animals were injected via the femoral vein with ^{3}HTdR, 6.0 Ci/m*M*, 1 μCi per g of body weight. One hr after ^{3}HTdR injection two 1 cm pieces of ileum were removed from an area 5 cm proximal to the ileocecal junction, fixed in Carnoy's solution for 24 hr, then stored in 70% ethyl alcohol.

Tissue was hydrolyzed in 1 N HCl and stained with the Feulgen reaction. Fifty crypts were isolated by microdissection in triplicate, placed in a counting vial to which a liquid scintillation mixture of toluene, PPO (5 g/liter) and DMPOPOP (0.3 g/liter) was added (1). After being dark adapted for 12 hr, the vials were counted to 10,000 counts in a Beckman liquid scintillation spectrometer, and dpm obtained by entering the data into the "CBLIQUID" computer program (CBLIQUID program obtained from C. Bischof and F. Kirchner, Radiation Research Laboratory, University of Iowa, Iowa City, IA) which calculated dpm from cpm and external standard channels ratio data.

Since one purpose of this investigation was

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E I. CHARACTERISTICS OF LABORATORY RATS USED BY REPRESENTATIVE INVESTIGATORS IN THE STUDY OF INTESTINAL EPITHELIAL CELL KINETICS.

nvestigator	Stock	Sex	Original weight	Interval between resection and sampling
Crocker (5)	Sprague-Dawley	Male	300–350 g	2 months
& Booth (3)	Wistar-Firth	Female	150–220 g	6 months
)	Albino Wistar	Male	143–183 g	No resection
t Osborne (2)	Holtzman	Male	250–300 g	2 months
)	Holtzman	Male	"adult"	No resection
l. (7)	Glaxo-Wistar	Male	250 g	2 months

II. COMPARISON OF PARAMETERS MEASURED FOR SIGNIFICANT INFLUENCES OF AGE, STOCK OF RAT, AND PERCENTAGE OF INTESTINE RESECTED.[a]

	Influences					
			Percent resection			
ieters ured	Stock (Sprague-Dawley vs Holtzman)	Age (70 g vs 250 g)	T	10	50	80
pt	>0.500	<0.001	>0.500	<0.500	<0.100	<0.050
	<0.500	<0.001	>0.500	>0.500	>0.500	<0.500
ig	<0.100	>0.030	>0.500	>0.500	<0.100	<0.010
it	<0.300	<0.400	<0.200	<0.200	<0.020	<0.050
	<0.500	<0.001	<0.400	<0.200	>0.500	>0.500

atries are probabilities of means being equal.

rmine whether dpm/labeled nucleus vas constant throughout the various l treatment groups, individual Feulgen crypts were "squashed" to spread the a monolayer, dipped in Kodak NTB · track emulsion, and exposed for a of 7 days. The slides were then devel- ad 10 crypts per animal scored for the r of labeled cell nuclei. The values for ypt were then divided by the values N/crypt to obtain the values of N. If the values of dpm/LN were it, the dpm per piece of tissue was ional to the number of proliferative the piece of tissue.

· 24 hr in fixative, a small piece of approx. 30 mg) was removed, dried, d, and solubilized in 0.5 cc Soluene rd Instrument Co., Downers Grove, nen 15 cc of the liquid scintillation ig mixture was added, the samples as- or radioactivity as noted above, and a converted to dpm/mg.

es of dpm/mg tissue were divided by ues for dpm/crypt (utilizing compa- ssue) to yield crypts/mg values.

dard error of the means were calcu- r all data, and an Analysis of Variance) test for differences within treatment and between treatment means.

lts. After appropriate statistical comparisons of the data for the variables examined, no significant differences ($P > 0.05$) due to rat stock were seen in the response of the crypt epithelium to resection (see Table II and Figs. 1–4).

The values of dpm/crypt, crypts/mg, and LN/crypt were increased significantly ($P < 0.05$) in the 80% resected group (Table II and Figs. 1, 3 and 4).

Age was also a factor which influenced some of the parameters. The values for dpm/crypt, dpm/mg, crypts/mg, and dpm/LN were consistently higher for the juveniles of both stocks (Figs. 1, 2, 3, and 5).

Discussion. Holtzman rats were used in this study because the response of this stock to the stimulus of intestinal resection is marked and has been well documented in this laboratory (1, 2). The Sprague–Dawley rat was chosen because it was the only other stock in which enough large animals could be obtained in a reasonable length of time. Since supply was a problem, it was fortunate that the cell kinetics from both stocks were the same.

No differences were noted in response to resection which could be attributed to the stocks of rats used (Table II and Figs. 1–4). This is not surprising when one considers that the Holtzman stock originally derived from the Sprague–Dawley stock some years ago.

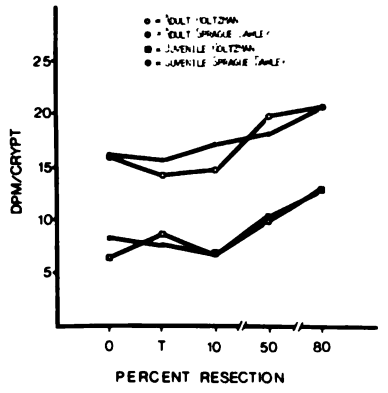

FIG. 1. The influence of percentage of intestine resected on the proliferative activity per crypt.

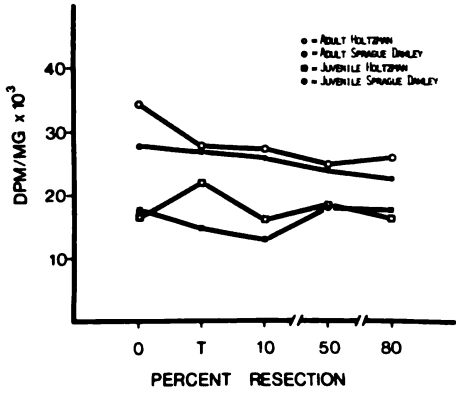

FIG. 2. The influence of percentage of intestine resected on the proliferative activity per milligram of tissue.

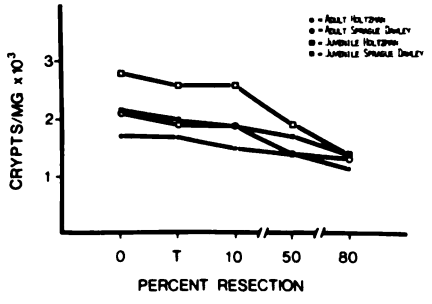

FIG. 3. The influence of the percentage of intestine resected on the number of crypts per milligram of tissue.

As shown in Fig. 4, dpm/labeled nucleus increased with animal age. This finding is very important since an experimenter using animals of different ages in studies involving the uptake of labeled thymidine, should take into account that cells synthesizing DNA do not incorporate the labeled precursor independent of age. It should also be noted from Table II that the degree of resection had no significant ($P < 0.05$) effect on the uptake of ^{3}HTdR per labeled cell nucleus. This is in agreement with the literature (1, 2).

Table II also shows that LN/crypt do not change with age, while dpm/crypt, dpm/mg, and crypts/mg do change. Therefore there must have been an increase in the uptake of ^{3}HTdR per intestinal epithelial cell nucleus in the older rats, in agreement with our findings of increased dpm/labeled nucleus with age.

Since there is no increase in chromosome material per nucleus with age, one possible explanation of the observed increase in uptake of ^{3}HTdR by the intestinal epithelial cells of adults is that there is more ^{3}HTdR available per cell nucleus due to fewer cells synthesizing DNA, i.e., the juvenile animals were in a period of rapid growth in which most tissues of the body were proliferating at

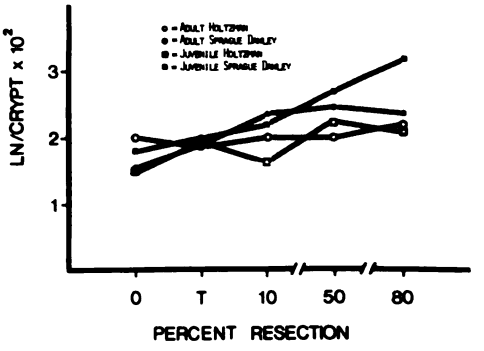

FIG. 4. The influence of the percentage of intestine resected on the number of labeled nuclei per crypt.

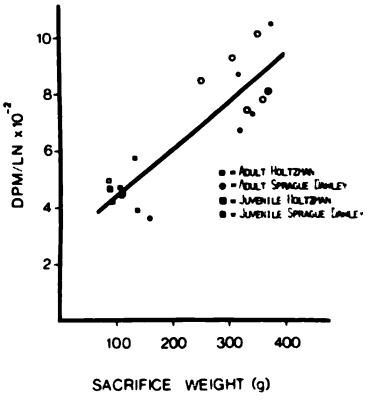

FIG. 5. The influence of animal weight on the uptake of labeled DNA precursor per labeled nucleus.

er rate than the adults and thus had ells capable of incorporating the DNA sor. Since the ^{3}HTdR was given on a am of body weight basis, the adults ave received a larger percentage of the precursor on a per cell basis.

results of the present study agree with of Hanson, Osborne, and Sharp (1), und that the magnitude of the response ction was dependent upon the percent- intestine removed, with no significantly nt response from normal noted when an 40% of the combined length of the and jejunum was removed, and with a section causing a maximum response. ıvenile rats showed a greater percent ıe in the dpm/crypt than did the cor- ding adult members indicating a percentage of the juvenile crypt nuclei the S phase of the cell cycle.

ke (4) noted that while the number of in the rat small intestine increases with ıe number of villi remains the same, he villi themselves become larger. This ; that the absolute numbers of crypts ıe with age to meet the demands of the ng villi for cells. This, added to an ıed functional demand per unit area by massive resection increases the pro- ve rate even higher, as was seen in the ıercent change in the juveniles of the ocks. The adults, which have reached where the crypt/villus ratio has essen- stabilized (4), showed a smaller per- e increase (Fig. 1).

e there was a marked increase in the rypt values for the juveniles of both and Hanson *et al.* (1) have shown that s a thickening of all intestinal wall nents after resection, with the degree kening increasing as the amount of removed is increased, the fact that ıg of intestine did not rise in the juve- nile age group is reasonable. This also explains why the values of dpm/mg of intestine fall in the adult age groups of both stocks of animals; the dpm/crypt was not rising in the adults as rapidly as it had in the juveniles and the thickening of the intestinal wall components increased at a greater rate, thus reducing the value of dpm/mg.

The values for crypts/mg were obtained by dividing dpm/mg by dpm/crypt. Again, on the assumption that all intestinal wall components increased in proportion to the degree of resection, it was reasonable for the values of crypts/mg to decrease as the percentage of intestine resected increased. In the juveniles of both rat stocks, the values for crypts/mg probably were consistently higher in young rats (which initially have smaller small intestines than the adults) than in their adult counterparts because the ability of their intestinal mass to increase was greater than the rate of increase of the crypts.

The larger percent decrease in the level of crypts/mg in the juvenile groups seems to be due to the larger increase in thickening of the intestinal wall components.

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Effects of Dopamine, Norepinephrine, and Serotonin on Plasma Prolactin Levels in Ovariectomized, Pituitary-Grafted Rats[1] (40100)

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The ability of the catecholamines to affect prolactin (Prl) release from the anterior pituitary (AP), both *in vitro* and *in vivo*, has been studied. Shaar and Clemens (1) demonstrated that norepinephrine (NE) and dopamine (DA), when administered *in vitro* in amounts not greater than that found in the hypothalamus, inhibited Prl release. This suggests a direct effect of these amines on the AP. This confirmed earlier *in vitro* work (2, 3), which indicated that several concentrations (10^{-6} M–10^{-4} M) of the catecholamines could inhibit Prl release. Kamberi *et al.* (4), using an *in vivo* system, concluded that neither DA nor NE affected Prl release by a direct action on the AP. They suggested that the effects of the catecholamines on Prl release were mediated through the hypothalamic–hypophyseal complex, with DA being more effective than NE in reducing plasma Prl. In direct contrast to this, Takahara *et al.* (5) demonstrated a lowering of plasma Prl following injection of NE or DA into the hypophysial portal system, indicating a direct effect of these amines on the AP. Blake (6) further demonstrated that DA, when infused intravenously, could significantly lower basal plasma Prl levels and block the spontaneous surge of Prl in plasma seen during proestrus. He could not conclude, however, whether the inhibitory effects of DA were directly on the AP or mediated through the median eminence of the hypothalamus.

Serotonin (5-HT), on the other hand, is increasingly implicated as a stimulator of Prl release. Lawson and Gala (7) demonstrated an increase in Prl release following systemic administration of 5-HT in ovariectomized, estrogen treated rats. 5-HT, injected into the third ventricle, also has been shown to stimulate Prl release (8). Parachlorophenylalanine, a drug used to chronically block 5-HT synthesis, inhibited the suckling induced rise in plasma prolactin (9). This drug also was found to decrease serum Prl levels in estrogen primed, ovariectomized rats (10). MacLeod, using an *in vitro* system, has shown no direct effect of 5-HT on Prl secretion from the AP (11).

The present experiments were designed to investigate further if amines have a direct effect on the AP secretion of Prl. Experiments utilized the ovariectomized rat with intact pituitary and an additional AP transplanted under the kidney capsule.

Materials and methods. Female, Sprague–Dawley rats (Laboratory Supply, Indianapolis, IN) were housed in a temperature (25 ± 2°) controlled room with lights on from 0500 to 1700 hr. Rats were given free access to rat chow and water. All rats were bilaterally ovariectomized. One week following ovariectomy, donor rats were killed by decapitation, the anterior lobe was separated from the posterior lobe and immediately transplanted under the right kidney capsule of the host rats (1 AP/rat). One week following pituitary transplantation, an indwelling aortic cannula was inserted via the right carotid artery under ether anesthesia using the method of Gay *et al.* (12). This cannula was then used for both the infusion of biogenic amines as well as for blood sampling the next day in awake and unrestrained rats.

The following compounds were utilized for infusions: L-norepinephrine bitartrate (lot number 510-1940) and dopamine HCl (lot number 930-1370) from Sigma Chemical, St. Louis, MO, and serotonin creatinine sulfate complex hydrate (lot number 901192) from Calbiochem Inc., Los Angeles, CA. Each amine was dissolved in a phosphate buffered saline (PBS)-ascorbic acid solution (0.02% ascorbic acid). The concentration of the biogenic amines in solution was 1 μg per 10 μl PBS-ascorbic acid adjusted to a final pH of 5.5 with 0.1 N HCl. This PBS-ascorbic acid solution, devoid of any biogenic amines, was utilized as a control solution at pH 5.5. All

[1] This research was supported in part by Public Health Service Grant Nos. HD06907 and GRS 531174.

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solutions were prepared fresh immediately before use and were kept on ice throughout the infusion procedure. In addition, all solutions were used only once; fresh solutions were made for each infusion.

Infusions were carried out with the aid of an LKB peristaltic pump (LKB-Produkter, Bromma, Sweden). The rate of infusion was kept constant at 10 μl per min throughout the experiment except for 20–30 sec interruptions when blood was withdrawn. A preinfusion blood sample was taken to establish basal Prl levels and the infusion was then begun. Samples were taken at 5, 10, 20, 30, 40, 50 and 60 min following the commencement of infusion. The infusion was terminated at 60 min and a final blood sample was taken 60 min later.

The Prl concentration of all blood samples was determined via a double antibody radioimmunoassay for rat prolactin (13). Purified rat Prl from NIAMDD was used for iodination and NIAMDD-RP-1 was used as a reference standard. Antibodies against rat prolactin were obtained from J. D. Neill (Emory University). A two-way analysis of variance and Dunnett's *t* test were used to compare plasma Prl levels between control and amine infused groups, as well as between the preinfusion sample and the postinfusion samples within each group.

Results. The results of this study are shown in Table I. The PBS infused homografted group showed a significantly higher basal plasma Prl level than the PBS infused group without any AP transplants. Assuming the half life is the same for Prl secreted from each pituitary site, at least 85% of plasma Prl may be due to the AP transplant. Infusions of both NE and 5-HT had no significant effect on Prl secretion when compared both to the PBS infused homografted group and to the preinfusion level within each respective group. Dopamine, however, was able to suppress Prl release by 5′ of infusion, and this decreased plasma Prl level was significant by 20′ when compared to the preinfusion levels. When compared to the PBS infused, homografted controls, plasma Prl levels were significantly suppressed after 5′ of infusion. By 40′ of DA infusion, plasma Prl levels were at their minimum, and remained basal until the infusion was stopped. This plasma Prl level was not significantly different from the levels seen in the PBS infused animals without pituitary transplants. After 1 hr following the cessation

TABLE I. EFFECT OF INFUSION OF DA, NE, AND 5-HT ON PLASMA PRL LEVELS IN OVARIECTOMIZED-AP TRANSPLANTED RATS.

	Prolactin (ng/ml)[b]								
Treatment	Pre[c]	5′	10′	20′	30′	40′	50′	60′	120′
OVX No Transplant PBS (5)[a]	16.5 ±9.2	13.1 ±5.9	7.4 ±1.5	12.9 ±4.0	7.8 ±1.1	9.1 ±1.4	10.9 ±4.1	7.8 ±0.1	9.4 ±0.5
OVX AP Transplant PBS (9)	93.3 ±11.7	94.1 ±11.1	85.2 ±9.5	94.8 ±9.1	80.4 ±7.9	90.2 ±8.5	84.6 ±6.4	82.2 ±6.2	94.9 ±6.8
OVX AP Transplant DA (7)	78.3 ±13.7	56.2 ±12.9[d]	43.9 ±13.9[d]	31.1 ±8.9[d, e]	23.9 ±7.9[d, e]	20.2 ±4.6[d, e]	20.6 ±4.6[d, e]	19.8 ±4.5[d, e]	97.8 ±26.2
OVX AP Transplant NE (7)	83.0 ±17.9	81.0 ±20.0	81.3 ±21.3	65.0 ±14.5	67.7 ±17.5	88.8 ±24.7	79.2 ±20.4	98.2 ±28.0	96.4 ±26.8
OVX AP Transplant 5-HT (8)	82.6 ±12.4	79.6 ±9.6	81.1 ±11.9	83.3 ±11.2	80.4 ±8.6	85.8 ±10.4	77.4 ±8.7	86.3 ±12.4	75.0 ±8.2

[a] Number of rats per group.
[b] Mean ± SE of the mean.
[c] All infusions began after the first sample was obtained and ended at 60′.
[d] Significantly different from PBS infused, homografted controls ($P < 0.05$).
[e] Significantly different from preinfusion levels ($P < 0.05$).

of DA infusion, plasma Prl levels returned to their preinfusion level.

Discussion. Isolated AP homografts, placed under the kidney capsule, are known to secrete tonically large amounts of Prl, presumably due to the removal of the inhibitory influence of the hypothalamus (14). This source of Prl accounts for the large difference in plasma Prl seen between the PBS infused homografted group and the PBS infused nontransplanted group. The suppression of plasma Prl levels seen during DA infusion indicate the ability of DA to directly suppress Prl release from an AP graft, *in vivo.* This effect was pronounced and had a rapid onset. It had been previously shown that systemic intravenous infusions of DA could depress basal plasma Prl levels in the intact, cyclic rat (6). While it is likely that some of the suppression of Prl release observed in our work may be occurring in the intact pituitary, the amount of suppression seen certainly demonstrates an inhibitory effect of DA directly on the isolated pituitary graft. If Prl secretion from the intact pituitary were to completely cease and no suppression of Prl release from the AP graft occurred, the resultant decrease in plasma Prl levels would be expected to be far less than that observed. It has been shown that rats previously hypophysectomized have detectable plasma PIF levels whereas intact controls do not (15). An intact hypothalamic–pituitary axis was present in our model, preventing the release of detectable amounts of PIF into the systemic blood. Injections of L-dihydroxyphenylalanine (L-Dopa) have been shown to increase Prl release inhibiting activity of systemic blood in hypophysectomized rats, presumably through stimulation of hypothalamic PIF (16). The use of DA rather than L-Dopa in our studies, however, precludes the large scale movement of this amine into the hypothalamus. These results demonstrate the ability of DA to directly and dramatically suppress Prl secretion from the anterior pituitary, *in vivo,* and are consistent with the view that DA may be PIF. However, this study does not eliminate the possibility that DA normally acts by stimulatmg release of another kind of PIF from the hypothalamus. These results also provide an animal model which is useful for studying agents which have a direct action at the AP on Prl secretion.

Norepinephrine at a dosage of 1 μg per min appeared to exert no effect on Prl release from either the pituitary graft or the intact pituitary. Higher doses were not used because of possible systemic effects of the NE. This seems consistent with the findings of Blake (6), who suggested that the partial suppression of the Prl surge on the afternoon of proestrus in the intact rat seen after two hours of systemic infusions of NE was a nonspecific effect. Serotonin also appeared to exert no effect on either pituitary. These findings are in agreement with the *in vitro* work of MacLeod, who found no direct effect of 5-HT on an isolated anterior pituitary (11). This does not refute the findings of those who observed an increase in Prl release following systemic injection of 5-HT (7), since 5-HT was administered in that study to estrogen-treated ovariectomized rats as a massive bolus injection. No steroids were administered in the present study.

Summary. Chronically ovariectomized rats with intact pituitary glands were implanted with anterior pituitary homografts under the kidney capsule. Rats which received a transplanted anterior pituitary exhibited significantly higher basal plasma prolactin levels than rats receiving no transplants. Rats received systemic infusions of norepinephrine, dopamine, and serotonin. Only dopamine was capable of significantly suppressing prolactin secretion from the pituitary homograft. Norepinephrine and serotonin had no effect on plasma prolactin levels. These results demonstrate the direct effect of dopamine on prolactin release by the anterior pituitary and are consistent with the view that dopamine may be PIF.

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Protective Action of Methylprednisolone on the Isolated Perfused Rat Heart Following Severe Hypoxia[1,2] (40101)

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In 1953, Johnson and coworkers (1) suggested that cortisone treatment limited the extent of myocardial damage after myocardial infarction. In their study these investigators found that low doses of cortisone diminished the size of experimental myocardial infarction. Since this early report, there have been conflicting reports regarding the efficacy of corticosteroids in attenuating the deleterious effects of myocardial ischemia (2). More recent studies (3, 4) have indicated that treatment of animals with steroids can limit the size of a myocardial infarction induced by experimental coronary occlusion. Ehbid *et al.* found that large doses of hydrocortisone given intravenously caused regression of abnormal S-T segment elevations of the electrocardiogram both in experimental myocardial infarction in dogs and in recent myocardial infarction in humans (5). More recently Libby *et al.* (3, 7) have studied the effect of large doses of hydrocortisone on the size of experimental myocardial infarction. By separate measurements of electrocardiographic, enzymatic and histologic criteria, these investigators found that corticosteroids decreased myocardial ischemic injury. Furthermore, it was also found that pharmacologic doses of hydrocortisone helped prevent subsequent necrosis even when given as late as 6 hr after the experimental coronary artery occlusion. These studies, although determining myocardial injury by monitoring cardiac enzyme activity and electrocardiographic changes which occur after a completed infarction, have not addressed themselves to evaluating the potential benefit of corticosteroids in enhancing the functional performance of the heart after an hypoxic or ischemic insult. However, Busuttil and coworkers, using an intact canine preparation, demonstrated that hearts of methylprednisolone pretreated dogs exhibited significantly better cardiac performance after aortic conclusion than did controls (6). The question still remained concerning the salutory effect of steroids on hearts that have been exposed to hypoxia. To this end, the present study was designed to determine whether pretreatment with a pharmacologic dose of the glucocorticoid, methylprednisolone, affords protection to the hypoxic isolated perfused rat heart and enhances the recovery of the heart during a period of reoxygenation.

Methods and materials. In this study hearts were obtained from male Sprague-Dawley rats (250–300 g) pretreated (ip) with either methylprednisolone sodium succinate 30 mg/kg 18 hr and 1 hr prior to sacrifice or the vehicle of methylprednisolone consisting of benzyl alcohol in 0.9% NaCl. The methylprednisolone sodium succinate was a generous gift of the Upjohn Company.

Coronary perfusion was conducted with an Anderson perfusion apparatus that contained modified Tyrode's solution equilibrated with 95% O_2 and 5% CO_2 (pO_2 450–500 mm Hg) and maintained at 37° and pH 7.4 ± 0.05. The ionic composition of the Tyrode's solution in millimoles per liter was NaCl, 120; KCl, 5; $CaCl_2$, 4; $MgSO_4$, 0.6; $NaHCO_3$, 30; KH_2PO_4, 0.6 and glucose 5.0. An hypoxic perfusion medium (pO_2 10–20 mm Hg, pH 7.4 ± 0.05) was produced by gassing an identical Tyrode's solution with 95% N_2 and 5% CO_2 for 30 min. Both oxygenated and hypoxic perfusion media were set in a parallel system so that with the turn of a stopcock, either medium could be used for coronary perfusion. In order to determine the effects of the lack of an exogenous energy substrate on cardiac performance, one series of vehicle-treated and steroid-treated hearts was perfused with glucose-free Tyrode's.

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In all experiments, perfusion pressure was maintained constant at approximately 55 mm Hg. A calibrated Grass Ft 0.03 force displacement transducer, connected to the heart by a silk suture attached to a hook placed into the cardiac apex, was used to measure force of contraction. This transducer was mounted on a movable assembly so that sufficient tension could be placed on each heart to place the resultant contraction at the peak of its length-tension curve. Contraction was recorded as grams of developed tension on a Grass model 7 polygraph. Heart rate was maintained constant throughout the experiment by constant pacing at a rate 20% above the intrinsic rate by square wave stimuli (4-8V, 4 msec duration) delivered from an E and M stimulator.

Perfusion of rat hearts was conducted in the following manner. Hearts in vehicle-pretreated and methylprednisolone-pretreated groups were equilibrated for a period of 12–15 min with oxygenated Tyrode's solution (pH 7.4, 37°), then perfused for 3 or 5 min with hypoxic medium, and finally reperfused with oxygenated Tyrode's for 5 or 10 min to determine recovery from hypoxia. Hearts perfused with glucose-free Tyrode's solution were exposed to hypoxic medium for 3 min and recovered for 5 min and hearts perfused with Tyrode's solution containing 5 m*M* glucose were exposed to hypoxia for 5 min and recovered for 10 min.

These periods of hypoxic exposure were selected after it had been first determined that an hypoxic insult of this duration would depress myocardial function to approximately 10% of control. Longer periods of hypoxia usually precluded recovery while shorter periods did not provide a sufficient insult to consistently evaluate recovery.

Results. Table I summarizes the effects of hypoxia on force of contraction of the isolated rat heart perfused with glucose containing Tyrode's solution. There is no significant difference in force of contraction between hearts of vehicle-treated and methylprednisolone-treated animals. Within 1 min of hypoxic perfusion there is a significant reduction in the force of contraction (grams of developed tension) of hearts of both vehicle-treated and methylprednisolone-treated rats. This decline in developed tension progresses in both groups of hearts to a value of 2.2 g and 2.9 g in the vehicle-treated and methylprednisolone-treated groups, respectively, at 5 min of hypoxic perfusion. This represents an approximate 90% reduction in force of contraction in both of these groups.

Also shown in Table I is the force of contraction produced by the hearts during the period of recovery (reoxygenation). Throughout the reoxygenation period hearts from methylprednisolone-treated rats develop more tension than do hearts from vehicle-treated animals. Moreover, the force of contraction is significantly greater ($P < 0.05$) in the former group at 3, 5, 7 and 10 min of reoxygenation. By 10 min of reoxygenation the methylprednisolone-treated group develops 22.2 ± 1.1 g of tension (approximately 100% of the prehypoxic value) while the vehicle-treated group only develops 10.5 ± 0.84 g of tension (approximately 54% of the prehypoxic value) at this same time period.

In Table II is shown the effects of hypoxia on the force of contraction of the isolated rat heart perfused with glucose-free Tyrode's solution. The fall in developed tension during hypoxia is qualitatively and quantitatively similar in both groups of hearts in this series of experiments. By 3 min of hypoxic perfusion, hearts from vehicle-treated and methylprednisolone-treated groups develop 0.63 ± 0.13 and 1.0 ± 0.43 g of tension, respec-

TABLE I. EFFECT OF METHYLPREDNISOLONE AND HYPOXIA ON FORCE OF CONTRACTION IN ISOLATED RAT HEARTS PERFUSED WITH TYRODE'S SOLUTION CONTAINING GLUCOSE.[a]

	Contractile force (grams tension)	
Time periods	Vehicle-treated	Methylprednisolone-treated
Prior to Hypoxia	19.6 ± 1.2	22.8 ± 1.3
Hypoxia		
1 min	7.3 ± 0.81	9.3 ± 0.63
3 min	3.3 ± 0.44	4.6 ± 0.60
5 min	2.2 ± 0.26	2.9 ± 0.31
Reoxygenation		
1 min	6.1 ± 1.1	10.4 ± 0.91[b]
3 min	11.4 ± 1.4	18.3 ± 0.77[b]
5 min	12.3 ± 1.8	22.1 ± 1.2[b]
7 min	11.5 ± 1.6	23.4 ± 1.0[b]
10 min	10.5 ± 0.84	22.2 ± 1.1[b]

[a] 5 m*M* glucose added to Tyrode's perfusion medium. Each value represents the mean ± SEM; $n = 7$ for steroid-treated group; $n = 7$ for vehicle-treated group.

[b] Indicates significant difference ($P < 0.05$) when methylprednisolone-treated group is compared to vehicle-treated group.

TABLE II. EFFECT OF METHYLPREDNISOLONE AND HYPOXIA ON FORCE OF CONTRACTION IN ISOLATED RAT HEARTS PERFUSED WITH GLUCOSE-FREE TYRODE'S SOLUTION.[a]

Time periods	Contractile force (grams tension)	
	Vehicle-treated	Methylprednisolone-treated
Prior to Hypoxia	14.4 ± 0.85	15.0 ± 1.6
Hypoxia		
1 min	3.6 ± 0.54	5.3 ± 0.33
2 min	1.6 ± 0.37	3.7 ± 0.67
3 min	0.63 ± 0.13	1.0 ± 0.43
Reoxygenation		
1 min	4.7 ± 0.48	9.3 ± 0.62[b]
2 min	6.0 ± 0.55	10.9 ± 0.90[b]
3 min	6.3 ± 0.25	12.1 ± 1.3[b]
5 min	5.0 ± 0.20	12.6 ± 1.2[b]

[a] Tyrode's solution did not contain glucose. Each value represents the mean ± SEM; $n = 4$ for steroid-treated group; $n = 4$ for vehicle-treated group.

[b] Indicates significant difference ($P < 0.05$) when methylprednisolone-treated group compared to vehicle-treated group.

tively, both approximately 5% of the prehypoxic values. During the reoxygenation period, hearts from methylprednisolone-treated rats develop significantly more tension ($P < 0.05$) than do hearts from vehicle-treated rats. The methylprednisolone-treated group regains a maximum force of contraction of 12.6 ± 1.2 g (84% of the prehypoxic value) whereas the vehicle-treated group attains a force of contraction equivalent to 6.3 ± 0.25 gm (44% of the prehypoxic value) at this same time period.

Discussion. The results of this study clearly demonstrate that pretreatment with a pharmacologic dose of the glucocorticoid, methylprednisolone, improves the functional recovery of the isolated hypoxic rat heart during a period of reoxygenation. The fact that the capacity of the heart to recover after hypoxia is better preserved in the methylprednisolone-treated group suggests that this steroid prevents some of the deleterious effects that are produced by hypoxia.

The precise mechanism by which methylprednisolone enhances cardiac recovery of the hypoxic rat heart is not clear, however, the protective mechanism may be related to the ability of methylprednisolone to reduce the amount of cardiac cellular damage as has been reported by other investigators (3, 4, 7). One of the primary causes of cellular damage during hypoxia is the disruption of lysosomal membranes with a release of acid hydrolases into the cytoplasm of the cell (8). Glucocorticoids have been shown to stabilize lysosomal membranes thus making them more resistant to an hypoxic stress (9). This lysosomal membrane stabilization would be expected to result in a decreased cardiac cellular autolysis and in an improved cardiac performance upon reoxygenation.

Although glucocorticoids have been reported to increase glucose transport and thus enhance the intracellular flux of this energy source (10), the present study does not support this process as the mechanism whereby methylprednisolone affords protection to the hypoxic heart. The data presented herein illustrate that methylprednisolone protects the isolated rat heart even when glucose was omitted from the perfusion medium. However, it was noted that hearts perfused with glucose containing Tyrode's solution did perform better during and after the hypoxic insult. Exogenous carbohydrates supplied to isolated hearts have been shown to help maintain available levels of high energy phosphates (11). Therefore, the improved contractile performance during hypoxia seen in these hearts perfused with 5 m*M* glucose appears to result from the availability of exogenous glucose. However, the improved contractile performance due to increased availability of glucose does not explain why the hearts of steroid-treated rats perfused with glucose-free medium performed better than vehicle-treated hearts. Regardless of the presence or absence of glucose, methylprednisolone produced this protective effect which suggests that the protective effect of this hormone may be unrelated to increased utilizable energy sources accessible to the heart.

Other protective effects of corticosteroids which have been proposed include: (a) decreases in peripheral vascular resistance (12), (b) prevention of the formation of a myocardial depressant factor (MDF) (13) and (c) increases in cardiac output, coronary blood flow and cardiac efficiency (14, 15). Since the experimental design of the present study has excluded such extracardiac factors as MDF and peripheral vascular resistance it appears that the cardiac protection seen in these ex-

periments was not due to a modulation of these factors. The above reports which have suggested that steroids exert a direct protective effect on the heart have been controversial. Lefer (16) was unable to elicit a positive inotropic effect in the intact cat heart after 20 mg/kg of methylprednisolone. The data from our study support this finding in that methylprednisolone did not exert a positive inotropic effect on the isolated rat heart under control conditions. This is further evidenced by the similar force of developed tension in hearts of both vehicle-treated and methylprednisolone-treated rats. Furthermore, although not shown, there were no differences in total coronary blood flow between the two groups of hearts suggesting that a change in total coronary blood flow was not the mechanism for the enhanced performance in the methylprednisolone pretreated group. However, no measurements were made testing the possibility of a redistribution of coronary blood flow which may have benefited the myocardium.

Although we have not elucidated the precise mechanism for the protective action of the glucocorticoid, methylprednisolone, on the hypoxic heart, it has been shown that this steroid does attenuate some of the deleterious effects of hypoxia and thus enhances the functional recovery of the hypoxic heart.

Summary. The isolated rat heart was studied to determine if pretreatment with the glucocorticoid, methylprednisolone (30 mg/kg), both afforded protection to the heart during a period of hypoxia and improved recovery during reoxygenation. Hearts from rats treated with methylprednisolone sodium succinate exhibited significantly better force of contraction during the period of reoxygenation than did hearts from vehicle-treated rats. This improved recovery of the steroid-treated group was seen in the presence or absence of glucose and was not related to a steroid-induced positive inotropism or enhanced total coronary flow. However, the possibility exists that methylprednisolone pretreatment was associated with a favorable redistribution of coronary blood flow and as such produced a protective effect on the hypoxic heart.

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Estimation of Plasma Half-Lives of Vasopressin Analogs from Vasopressor Responses by Curve-Fitting (40102)

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Finding plasma half-lives by measuring peptide concentrations in plasma after injection or infusion has serious limitations when one wishes to study rapidly-eliminated peptides, such as vasopressin, in a small animal. The time needed to draw blood samples becomes an important source of imprecision. The volume of blood which one must take is also a limiting factor. One usually cannot repeat half-life measurements on one animal or compare two peptides in one animal, because this would require taking too much blood. If one could estimate plasma half-lives from a biological response one could avoid these limitations of direct plasma measurements.

In this paper we describe a method for estimating plasma half-lives by comparing the rate of decay of a biological response to the dose-response curve for the same peptide in the same rat. This method has been applied to arginine-vasopressin and two of its analogs using vasopressor responses in rats.

Materials and methods. Female rats weighing about 200 g were anesthetized with sc urethane (175 mg/100 g) and the trachea, carotid artery, and jugular vein were cannulated. After surgery, 1 ml of a 1 mg/ml solution of phenoxybenzamine (Dibenzyline, Smith Kline and French) was given sc. The carotid cannula was connected to a Statham pressure transducer and blood pressure was recorded on a Grass polygraph.

In the first experiment, which included nine rats, two rats were usually studied simultaneously. After their basal blood pressures became stable, one rat received arginine-vasopressin (AVP), the other, 1-deamino-arginine vasopressin (dAVP). Both peptides were diluted in 0.05 *M* acetic acid, 0.5% chlorbutanol solution and given in a series of 6 iv injections (2.5, 5, 10, 20, 40, 80 ng sequentially in order of ascending dose) at 15-min intervals. Injections were given rapidly. The total volume of injection plus wash did not exceed 140 μl. This volume of chlorbutanol solution given alone did not alter blood pressure. One hour was allowed to elapse after the last injection of the series. During this time, blood pressure returned to basal levels. The injections were then repeated with each rat receiving the other peptide in a series of injections identical to the previous series.

From the resulting blood pressure tracings, of which Fig. 1 is an example, tables of mean blood pressure vs. time were made for each dose for each rat. Mean blood pressure was measured at 2-min intervals beginning at the time of maximum response. The baseline blood pressure was taken to be the mean blood pressure immediately preceding the injection. Mean blood pressure was calculated using the formula: mean blood pressure = diastolic blood pressure + ⅓ (systolic blood pressure − diastolic blood pressure).

From the tabulated blood pressure data, log dose vs peak response graphs (as in Fig. 2) and response vs time graphs (as in Fig. 3) were made for each drug in each rat. All subsequent calculations were made with data from these tables or graphs. In a few rats, the response to dAVP did not decline in 15 min. The data from these rats were discarded.

The second experiment was similar to the first except that the rats were injected with arginine-vasotocin (AVT). Seven rats were each given two series of injections. The first series of six doses (3.75, 7.5, 15, 30, 60, 120 ng) was sequential as in Experiment 1. In the second series the six doses were given in a random order determined by assigning each dose a number generated by a random number generator and reordering the doses according to the assigned numbers. In this experiment, the rats were allowed to rest 15 min at basal blood pressure levels between successive injections.

AVP (376 vasopressor U/mg), dAVP (330

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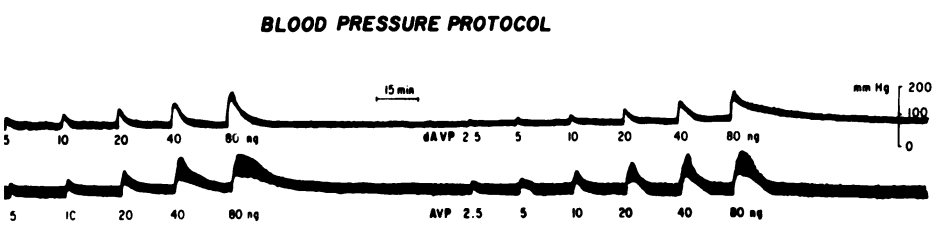
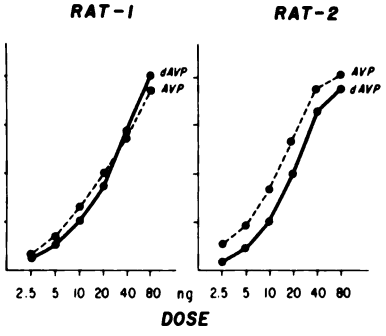


Blood pressure tracings, Rats 1 and 2. Rats were pretreated with phenoxybenzamine and given a series s of AVP and dAVP.



Log Dose vs Response. The ordinate records m blood pressure increments which occurred to a series of injections of 2.5, 5, 10, 20, 40, f AVP or dAVP.

ınd AVT (227 U/mg) were synthe- Maurice Manning and co-workers

nd dAVP were used initially to es- ıis method for estimating half-lives ɔonses because their half-lives had ʻiously estimated from direct plasma nents from the initial part of the disappearance from plasma follow- ıion of prolonged constant infusions ptides and had been found to differ ıtly (3).

. Inspection of the blood pressure from the first experiment showed higher doses of dAVP produced ısopressor responses than did doses which were equipotent in terms of ı responses in every rat (as, for ex- ı Fig. 1). The longer responses to ːre consistent with its longer half-life y estimated from infusion experi- . We then attempted to demonstrate pressor responses could be analyzed independent estimates of plasma . To do this we considered that the or response could be divided into ses: (1) a period during which pressure reaches a peak and the iv injection mixes in the plasma volume, (2) a period in which peptide levels fall toward threshold and the pressure declines, and (3) the time it takes for the vasopressor response to disappear completely after the plasma concentration has fallen below threshold levels. In a series of injections of increasing doses, we assume that phase 1 and phase 3 remain nearly constant in duration, but phase 2 lengthens. By comparing the time courses of responses to a series of injections, the influence of the drug half-life (which determines the length of phase 2) can thus be separated from the other components of the response.

In order to find a numerical value for each half-life, we made two other assumptions. The first was that, in a given rat, equal elevations of blood pressure reflected equal plasma peptide concentrations, whether the elevation was the maximum response to an injection or occurred during the declining phase of the response to a larger injection. Development of peak pressure or the rate of decline may both lag behind the peak and decline phases of plasma peptide concentration. If these lag times remain constant they would not affect our estimates of half-life. The second was that the logarithm of the plasma peptide concentration fell linearly with time after the maximum response was reached. This would occur if the three routes of exit from the plasma, metabolic clearance, urinary clearance, and diffusion into the extravascular space, were all exponential processes.

To estimate half-life, a log dose-response curve (Fig. 2) was constructed from the maximum responses to a series of iv injections. If log plasma concentration falls linearly with time after iv injection the time course of the decay of the response to the largest injection of the series used to construct the log dose-

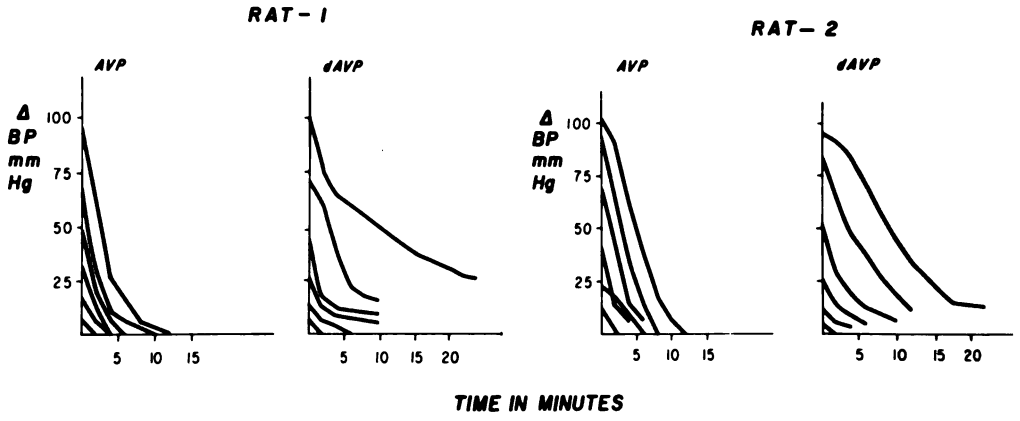


FIG. 3. Response vs Time. Each graph shows the time course of responses to a series of injections (2.5, 5, 1(40, and 80 ng) of AVP or of dAVP. The time at which the response reached a maximum is taken as time 0.

response curve is a continuous "unrolling" of the log dose-response curve. The two curves should be superimposable if a proper fixed interval between doublings of dose in the log dose-response curve is chosen.

Curve 1 (in Fig. 4) shows the decline in response as log dose (which is proportional to log plasma concentration) declines. If log plasma concentration fell linearly with time, the same response curve would be generated, but time would be the abscissa. Each successive point on Curve 1 represents the response to half the dose of the previous point. Halving of the plasma concentration is what would occur in one half-life. Curve 2 is the decay of the response after peptide injection. If the correct half-life is chosen, the points of Curve 1 can be fitted at half-life intervals on Curve 2. This curve-fit method involves fitting the middle four responses of the log dose-response curve (that is, the maximum responses to 5, 10, 20, and 40 ng, in the case of AVP and dAVP) on the response vs. time curve by postulating a fixed time interval between them (Fig. 5). The time interval which results in the best fit is taken to be the half-life. The fitting was done by trial and error. Usually we started by fitting responses to doses 3 and 4 exactly on the response vs time curve and used the time interval between the fitted points as our initial guess for the half-life. The interval was then adjusted as needed to bring responses to doses 2 and 5 into closer agreement with the time curve.

The decay of the response curves could be fitted quite closely in the middle range of the

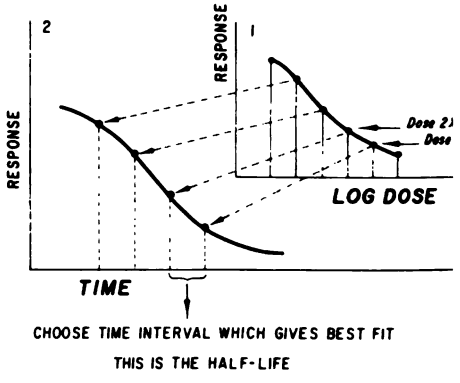


FIG. 4. Curve-fit Method. The middle four point the log dose-response curve are fitted on the respons time curve by postulating a fixed time interval betw the points. The time interval which results in the bes is taken to be the half-life.

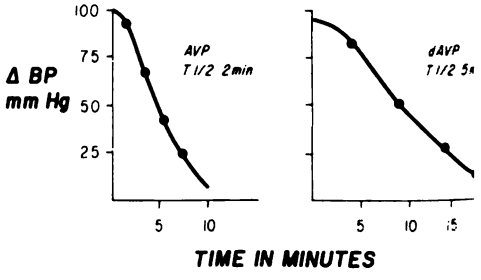


FIG. 5. The curve fit method applied to Rat 2. ' solid lines represent the decay of the vasopressor sponse to 80 ng of each peptide. The filled circles peak responses to single injections in the same rat. Th are plotted at the time intervals that approximate best fit to the decay curve.

se response curves. This suggests that sumptions that blood pressures during cline do reflect plasma peptide levels ıat these do decline exponentially in ınge are at least approximately true. prolonged infusions the measured ı concentrations of AVP and dAVP (3) decay at a single rate, but decay more y initially and then more slowly. From rates "early" and "late" half-lives can ıained. In the present single injection ments the rates of decay appear to be exponential and were about twice the ;orresponding to the "early" half-lives ted from infusion experiments.

f-lives obtained by the curve-fit method lividual rats are shown in Table I. Half- :stimated thus for AVP, dAVP, and ıre 2.6 ± 0.3 (SEM), 9.2 ± 1.4, and 4.0 min.

ussion. The 2.6-min half-life for AVP ted from the vasopressor response falls the range of previously published es- s based on measurement of plasma ıtrations following single injections in lly hydrated rats. These vary from 1.0 min (4). The variation among estimates sma half-lives following iv injections nably reflects differences in anesthesia, presence or absence of adrenergic blockade, the intervals at which plasma samples were obtained, and the limitations of the several assay methods. Data on the post-injection half-life of dAVP in rats are not available.

The half-lives obtained from vasopressor responses, 2.6 min for AVP and 9 min for dAVP, are shorter than, although proportional to, "early" half-lives measured directly from plasma concentrations after constant iv infusions, which are 4.5 min for AVP and 18.4 min for dAVP (3). The reason that postinfusion half-lives are longer may be that peptide accumulates in the extravascular space during infusions and diffuses back into the plasma after the end of the infusion. This would produce a longer half-life as estimated from the rate of decay of plasma peptide concentrations. When single injections are given, however, the early fall in plasma concentrations would reflect metabolic and excretory clearances and diffusion of peptide into the extravascular compartment. This would shorten plasma half-life. Thus neither infusion nor injection experiments necessarily yield a physiologically relevant value for the half-life of a peptide, one may overestimate, the other may underestimate. The plasma half-life of endogenous AVP would appear to depend, therefore, on whether it is released continuously, as if infused, or in spurts, as if injected iv.

In the first experiment, the order in which the peptides were given did not introduce a bias into the results (Table IA). In the second experiment, sequential dosing (low dose to high) did not yield half-lives which were significantly different from those obtained from responses to doses given in random order (Table 1B). When the half-life measurements for AVT were repeated on single rats, the average standard deviation was ±0.3 min. This is less than the standard deviation for measurements between rats (±0.9 min). This was calculated for the seven rats given AVT using a half-life for each obtained by averaging the "ascending" and "random order" half-life values. For comparison, using bioassay of plasma samples, Forsling et al. (5) found a half-life for AVP of 1.6 ± 0.8 (SD) min.

When using the curve-fit method, one must take into account the fact that repeated injections of high doses of peptide at close inter-

ıLE I. EFFECTS OF THE ORDER IN WHICH :PTIDES WERE ADMINISTERED ON HALF- ; (IN MINUTES) ESTIMATED BY THE CURVE-FIT METHOD.

ɔrder in which AVP and dAVP were injected.

AVP given 1st			dAVP given 1st	
AVP	dAVP	Rat	AVP	dAVP
1.5	12.0	2	2.0	5.0
2.0	12.0	3	2.5	6.0
3.5	6.0	8	2.0	8.0
3.5	6.0	13	4.0	18.0
2.5	10.0			
2.6	9.2		2.6	9.2
±0.4	±1.4		±0.5	±3.0

Γ administered in ascending and random orders of doses.

Rat	Ascending	Random
14	4.0	3.0
15	4.0	3.0
16	3.5	4.0
17	4.0	4.0
18	3.0	3.0
19	5.0	5.0
20	5.5	5.0
n ± SEM	4.1 ± 0.3	3.9 ± 0.3

vals can cause tachyphylaxis of the response. Before one obtains half-lives for any peptide by the curve-fit method, one must first find a dosing interval long enough to prevent tachyphylaxis. The interval varies with the peptide and must be found empirically.

An advantage of the curve-fit method is that it can separate components of the response. For example, if the plasma peptide concentration included a substantial contribution of peptide from the extravascular space at later times in the course of the response, concentrations would fall with a different "half-life" at later times. This would show up in the curve-fit method as a deviation of the later part of the decay of the response from the curve predicted from the log dose-curve. Also the curve-fit method separates the contribution of the slope of the log dose-response curve to the length of the response from that of the plasma half-life.

The log dose-response relationship influences the length of action in the following manner. If two drugs are similarly distributed and are eliminated at the same rate (that is, the fractions of total drug eliminated per unit of time are equal for the two drugs) and in the same manner, the drug with the less steep regression of response on log will be longer-acting, if one starts from the same degree of biological response for each drug. Since the concentration of each drug in the vicinity of the receptor would fall at the same rate, that drug which acts over a wider range of concentrations (that has a less steep log dose-response relationship) would have a more persistent action. Thus a long drug half-life is not the only factor which may produce a long response, and it is important to be able to separate the effect of drug half-life from the effect of other factors on the length of response.

Other investigators, such as Pliška (6), have measured the half-lives of responses in studies on vasopressor, antidiuretic and uterotonic responses to neurohypophysial peptides. Such measurements are easily made but cannot be readily interpreted in terms of plasma half-lives. Measurements of half-lives of responses would only yield reliable information on relative rates of elimination if all responses were of the same magnitude. Half-lives of responses clearly vary with the magnitude of the initial response (Fig. 3). Comparing half-lives of responses to different peptides would only relate to plasma half-lives if all the peptides had identical log dose-response relationships in all rats. The curve-fit method, although requiring certain unproven assumptions, would appear to provide more meaningful data on the rates of disappearance of peptides from the vicinity of the receptors, and, we presume, from the plasma, since this method factors the decay of the response by the log dose-response relation for each peptide in each rat. Thus variations in log dose-response relations among peptides and among rats do not influence the estimation of half-lives by curve-fitting.

We believe that half-lives estimated by curve-fitting better reflect actual plasma half-lives than do those estimated from the decay of responses alone. In the experiment illustrated in Fig. 5, for example, half-lives of responses to the highest doses of AVP and dAVP were about 5 and 9 min, respectively. These are about twice the half-lives estimated by curve-fitting. The shorter estimate of AVP half-life obtained by curve-fitting is in much better agreement with reported estimates based on actual measurements of plasma AVP levels following single injections (4).

To test directly the assumptions that we have made regarding the relation between vasopressor responses and plasma peptide concentrations would require simultaneous measurements of plasma peptides and vasopressor responses in individual rats. At present this would be technically difficult if not impossible. Nonetheless, we feel that curve-fitting offers substantial advantages over measurement of half-lives of responses alone as a simple and rapid means for estimating plasma half-lives of neurohypophysial hormones and their analogs. This method may also be applicable to studies on half-lives of other rapidly eliminated hormones and drugs.

Summary. Vasopressor responses by rats to graded doses of AVP, dAVP, and AVT were analyzed in an attempt to find a method for estimating plasma half-life from responses. The resulting "curve-fit" method indicated half-lives of 2.6 ± 0.3, 4.0 ± 0.3, and 9.2 ± 1.4 min for AVP, AVT, and dAVP, respectively, after single intravenous injections. The half-lives of two peptides can be compared in

one rat the "curve-fit" method. This method can also provide useful information concerning the relative contributions of the plasma half-life, the log dose-response relationship, and other factors, to the duration of the response.

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Calcium–Phospholipid–Phosphate Complexes in Mineralizing Tissue[1] (40103)

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Past work has shown that the acidic phospholipids are involved in tissue mineralization (1–3). Calcium–phospholipid–phosphate ($Ca–PL–PO_4$) complexes, containing the acidic phospholipids, phosphatidyl inositol (PI) and phosphatidyl serine (PS), were isolated from the long bones of developing animals (4). These complexes induced hydroxyapatite (HA) formation in metastable calcium phosphate solutions (5). The same study also showed that the uncomplexed acidic phospholipids when placed in metastable calcium phosphate solution, first formed a $Ca–PL–PO_4$ complex and then nucleated HA. The fact that more complexed lipids were found in the bones of developing as opposed to more mature animals further supports the thesis that such compounds are related to the onset of mineralization. The purpose of this study was to analyze further for complexed lipids in a number of hard and soft tissues in order to enlarge our view of the role of the $Ca–PL–PO_4$ complexes in mineralization.

Materials and methods. This investigaton was carried out on white New Zealand rabbits of ages 1–4 days (50–70 g), 4–6 weeks (0.9–1.4 kg), 12–16 weeks (1.5–1.8 kg), and older than 100 weeks (3.6–5.4 kg). The tissues studied were: cortex of long bones, epiphyseal regions of long bones (including articular cartilage, growth plate, and secondary ossification centers), skull bones (cranium and mandible), scapulae, marrow, adipose tissue, heart muscle, pooled leg muscles, ear cartilage, and incisors. All bones were scraped to remove the periosteum and marrow, ground in a liquid nitrogen-cooled colloid mill, washed in 0.05 M tris-hydroxy-methyl amino methane-HCl (Tris) buffer at pH 7.4 to remove blood, and then were lyophilized. Noncalcified tissues were frozen and minced with dry ice and then lyophilized. The ash weight (after heating to equilibrium weight at 600°), was determined for samples of each of the lyophilized tissues. Tissues of at least three different rabbits in each age (weight) group were analyzed separately; the analyses on each tissue specimen were performed a minimum of 3×.

Lipids were extracted from weighed dried tissue specimens using, (A) an earlier published method (4) and, (B) a new enzymedependent method described here for the first time. In method A each sample of tissue was sonicated in the presence of 2:1:1.5 chloroform:methanol:Tris buffer (30 ml/g). Total lipids, obtained after repeated sonications, were washed in dilute buffer, then dried under a stream of nitrogen and stored in vacuum. Repeated treatment of the total lipids with 2:1 ethanol:ether (v/v) solubilized the noncomplexed lipids leaving the complexed lipids as the residue.

In order to make certain that the sonication procedure (A) removed all lipids, certain tissue residues, remaining after the sonication-lipid extractions, were decalcified (5% EDTA) and then reextracted for lipid (6). The organic phase was then washed with water, dried under a stream of N_2 gas and stored in vacuum.

In method B the specimen was decalcified by dialysis against a 0.1 *M* pH 5 phthalate buffer (3 liter/g/day) for a 5-day period. The complex had previously been shown to be stable in acid above pH 4 (5). The remaining matrix, containing less than 10% mineral, was washed in water, lyophilized, and digested for 16 h at 37° in a Tris-buffered, pH 7.4 solution containing 1000 units each of collagenase and hyaluronidase per g of tissue (7). The lipids were then extracted from the remaining solid and aqueous phases by the addition of 2:1 chloroform:methanol so that the final volume ratio was 2:1:1 chloroform:methanol:buffer. After washing the organic phase with buffer, the total lipids were dried under a N_2 stream and the non-complexed lipids were isolated from the com-

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›y ethanol:ether extraction as in
A, above.
lated complexes were washed in 10^{-5}
› remove any traces of mineral. This
e did not change the composition of
›lexed lipids to any significant extent.
:ed lipids were then separated from
-complexed lipid impurities by two-
›nal thin layer chromatography (8)
ı the complexed lipids remained at
ı while the noncomplexed lipids mi-
he noncomplexed lipids were puri-
:hromatography on Sephadex G-25
›d under a nitrogen stream and
Aliquots of the complexed lipids
ı dissociated in formic acid and par-
in 2:1:1 chloroform:methanol: 1N
. The Ca (11) and inorganic PO_4 (12)
›f the organic phase were analyzed
trically. The phospholipid content of
›lexed and noncomplexed lipids was
by two-dimensional silica gel chro-
›hy (8) followed by analysis of the
› content of the individual spots (13).
P content of lipids extracted after
ılization was also determined, but
/idual phospholipids in these very
ctions were not identified.
es (0.3 mg) of each of the complexed
›lated from all of the tissues, as well
ı noncomplexed lipid samples, were
›pended in 10 ml of a metastable
phosphate solution (5) and the sus-
mixed for 7 days at room tempera-
y solid formed after 7 days was sep-
/ centrifugation (10 min at 2000 rpm)
Ca and PO_4 concentration of the
es measured by atomic absorption
›otometry (14) and colorimetry (12),
ely, for comparison with the com-
of the original metastable solution.
titic nature of all precipitates was
d by X-ray diffraction and chemical
(5).

r. Table I shows that for all ages of
studied, a Ca–PL–PO_4 complex can
ted from mineralized tissues, but not
›se tissues which do not normally
n the table the weight percentage of
›d lipid in the tissues is expressed in
/ays: (a) per total lipid (CL/TL), (b)
ineralized (i.e., organic) dry weight
G), and (c) per dry tissue weight
Also listed is the percentage of lipid P in the complex: (d) per total lipid P (CL-P/TL-P), and (e) per total acidic phospholipid P (CL-P/APL-P). The acidic phospholipids were phosphatidyl inositol, phosphatidyl serine, phosphatidic acid, and lysophosphatidyl inositol. Expressed in this manner it is evident that the complexed lipids were present in greatest amount in the most actively calcifying tissues (long bone, skull and scapula), and were very low in noncalcifying tissues (marrow, adipose, muscle, heart and ear cartilage).

There was no significant difference between the yield of complexed lipids obtained by the sonication method (A) and by the enzyme method (B). The yield of complexed lipid per total lipid in table I is the average yield for both extraction methods. In addition, independent of the method used, no additional phospholipid was released following EDTA decalcification of the extracted tissues.

The composition of several of the isolated complexes is shown in Fig. 1. Corroborating our earlier studies (4), all complexes isolated, in addition to those shown in the figure, have a Ca to total phosphate molar ratio of 1:1 and contained variable amounts of the acidic phospholipids and inorganic phosphate. In addition, each of the isolated complexes when placed in the metastable calcium phosphate solution yielded hydroxyapatite at the same rate. Figure 2 shows that the rate of change in composition of the metastable solution resulting from exposure to each of a variety of complexed lipid substrates was the same after the same time interval. The noncomplexed lipid fraction caused no change in the composition of these metastable solutions, (not shown), in the time period studied.

Discussion. Ca–PL–PO_4 complexes were absent from all nonmineralized tissues, even those rich in both lipid and Ca. This fact, plus the observation that complexed lipids are present in all mineralizing tissues, reaffirms the view that the complexes play some role in the calcification process. We have extended our earlier study (4) to show, with a greater number of tissue types and greater age range, that the highest proportion of complexed lipids, relative to a number of tissue parameters, is found in the younger, actively growing animals.

It was previously suggested (4, 5) that the

TABLE I. COMPLEXED LIPIDS OF MINERALIZED AND NON-MINERALIZED RABBIT TISSUES.

Tissue	Age[a]	CL/TL[b]	CL/ORG[c]	CL/T[d]	CL-P/TL-P[e]	CL-P/APL-P[f]
Long bone	1–4 day	16 ± 2	1.02 ± .09	0.65 ± .07	45 ± 5	73 ± 8
		$n = 9$	$n = 9$	$n = 9$	$n = 6$	$n = 3$
	1 Mo	11.5 ± .2	0.81 ± .03	0.34 ± .03	22.1 ± .7	45 ± 5
		$n = 12$	$n = 12$	$n = 12$	$n = 6$	$n = 3$
	4 Mo	7.6 ± .7	0.27 ± .03	0.087 ± .008	12 ± 2	14 ± 3
		$n = 12$	$n = 12$	$n = 12$	$n = 6$	$n = 3$
	Adult	4 ± 1	0.25 ± .04	0.078 ± .01	10 ± 2	10 ± 3
		$n = 12$	$n = 12$	$n = 12$	$n = 5$	$n = 3$
Skull bone	1–4 day	18 ± 2	0.37 ± .07	0.32 ± .09	33 ± 5	58 ± 6
		$n = 9$	$n = 9$	$n = 9$	$n = 6$	$n = 3$
	4 Mo	14 ± 2	0.20 ± .02	0.54 ± .05	21 ± 3	27 ± 3
		$n = 9$	$n = 9$	$n = 9$	$n = 8$	$n = 3$
	Adult	11 ± 2	0.12 ± .02	0.50 ± .04	15.4 ± 0.5	23 ± 1
		$n = 11$	$n = 11$	$n = 11$	$n = 4$	$n = 3$
Incisor	1–4 day	16.5 ± .2	0.44 ± .09	0.072 ± .005	6.8 ± .8	52 ± 4
		$n = 8$	$n = 8$	$n = 8$	$n = 3$	$n = 3$
	4 Mo	4 ± 3	0.19 ± .09	0.02 ± .01	9.9 ± .8	10 ± 2
		$n = 6$	$n = 6$	$n = 6$	$n = 3$	$n = 4$
	Adult	3 ± 1	0.1 ± .1	0.002 ± .001	6 ± 1	7 ± 1
		$n = 12$	$n = 12$	$n = 12$	$n = 3$	$n = 3$
Scapula	1–4 day	20.2 ± .3	0.59 ± .03	0.4 ± .1	47 ± 6	77 ± 5
		$n = 9$	$n = 9$	$n = 9$	$n = 5$	$n = 3$
	4 Mo	7.13 ± .06	0.6 ± .1	0.080 ± .007	23 ± 6	27 ± 3
		$n = 9$	$n = 9$	$n = 9$	$n = 5$	$n = 3$
	Adult	1.5 ± .4	0.3 ± .2	0.049 ± .009	6.2 ± .7	6 ± 2
		$n = 9$	$n = 9$	$n = 9$	$n = 3$	$n = 3$
Epiphyseal cartilage	1–4 day	13.9 ± .7	0.72 ± .03	0.20 ± .1	9 ± 1	10 ± 1
		$n = 10$	$n = 10$	$n = 10$	$n = 3$	$n = 5$
	4 Mo	6 ± 1	0.32 ± .09	0.14 ± .03	0.6 ± 1	1 ± 1
		$n = 9$	$n = 9$	$n = 9$	$n = 4$	$n = 5$
Marrow	Adult	0.020 ± .06	0.058 ± .004	0.058 ± .004	0	–
		$n = 9$	$n = 9$	$n = 9$	$n = 3$	
Adipose	Adult	0.021 ± .02	0.04 ± .02	0.04 ± .02	0	–
		$n = 9$	$n = 9$	$n = 9$	$n = 3$	
Leg muscle	4 Mo	0.03 ± .02	0.0022 ± .0005	0.0022 ± .0005	0	–
		$n = 9$	$n = 9$	$n = 9$	$n = 3$	
Heart	Adult	0.020 ± .005	0.002 ± .001	0.0022 ± .0005	0	–
		$n = 9$	$n = 9$	$n = 9$	$n = 3$	
Ear cartilage	1–4 day	0.00	0.00	0.00	0	–
		$n = 6$	$n = 6$	$n = 6$	$n = 3$	
	Adult	0.00	0.00	0.00	0	–
		$n = 6$	$n = 6$	$n = 6$	$n = 3$	

[a] Rabbit age; 1–4 days = 50–70 g, 1 Mo = 1.0–1.4 kg, 4 Mo = 1.5–1.8 kg, Adult = greater than 3.5 kg.

[b] CL/TL, wt. % complexed lipid per total lipid ±SE of measurement for n extractions.

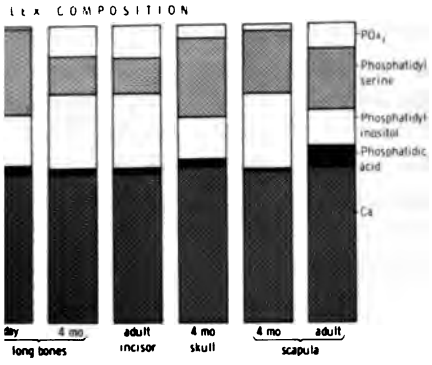
[c] CL/ORG, wt. % complexed lipids per demineralized (i.e., organic) dry wt. ±SE for n extractions.

[d] CL/T, wt. % complexed lipids per dry tissue wt., ±SE for n extractions.

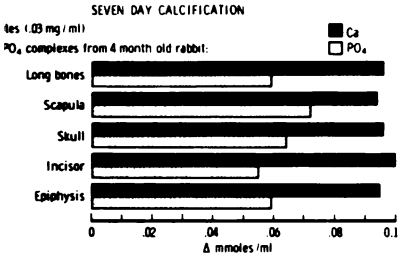
[e] CL-P/TL-P, % organic phosphorus in complexed lipids fraction relative to organic phosphorus in total lipid fraction, ±SE for the analysis of n complexes.

[f] CL-P/APL-P, % organic phosphorus in complexed lipids relative to organic phosphorus in total acidic phospholipids ±SE for the analyses of n complexes.

Ca–PL–PO_4 complexes may be components of the extracellular matrix vesicles known to be present in all mineralized tissues (15) and/or components of the membranes of calcifying cells (16). Wuthier has recently reported the isolation of such Ca–PL–PO_4 complexes from epiphyseal cartilage matrix vesicles in amounts constant per unit vesicle (17). Since the presence of extracellular matrix vesicles is associated with the onset of mineralization (15) it is not surprising that our greatest yields of complex were obtained from those tissues which are most actively forming new mineral.



1. The mean chemical composition (mole %) of PO_4 complexes extracted from a variety of calsues.



2. The effect, after 7 days (25°), of a series of ed lipid substrates, extracted from different tisthe level of Ca and PO_4 in a metastable Ca–PO_4 The composition of the original metastable was 1 m*M* Ca, 1 m*M* HPO_4, pH 7.5, 0.05 *M* l.

ies are underway in our laboratory to ine the distribution and the concentrathe complex in the zones of the epil growth plate. In addition, we are ; to determine whether Ca–PL–PO_4 xes are components of the proteolipids others (18–20) have isolated from lizing tissues and which have been to induce *in vitro* hydroxyapatite for-

mary. Calcium–phospholipid–phosomplexes have been isolated from mied tissues using a sonication-dependthod described previously and a new l which depends on the use of enzymes ove the nonlipid matrix from tissues were decalcified under mild condioth methods gave comparable results. mplexes were found to be constituents of all mineralized rabbit tissues; bone, calcified cartilage, and tooth. No such complexes could be isolated from the nonmineralized tissues; muscle, ear cartilage, bone marrow, adipose tissue. The proportion of Ca–PL–PO_4 is highest in those tissues which are involved in active mineralization, i.e., the younger tissues. All complexes isolated from mineralized tissues contain 50 mole % Ca, and variable amounts of the acidic phospholipids and inorganic phosphate. However, the Ca:total P molar ratio was always unity. All isolated complexes were shown to induce *in vitro* hydroxyapatite formation from metastable calcium phosphate solution and, thus, are believed to be associated with *in vivo* calcification.

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An Immunological Comparison Between Mouse α-Fetoprotein and the Uterine Estrogen Receptor (40104)

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Recent observations on the binding of estrogens to α-fetoprotein (AFP) have created considerable interest in the possible functional significance of this interaction. Both rat and mouse AFP bind estrone and estradiol-17β with a high affinity and specificity, suggesting that the interaction may be biologically significant (1–6). One suggestion is that AFP may protect certain tissues from exposure to high estrogen levels during critical stages of their development (7–10).

Another possibility is that AFP may in some way be involved in the action of estrogens in target tissues. A recent report by Uriel *et al.* (11) supports this possibility. These investigators demonstrated an immunological similarity between rat AFP and the uterine estrogen receptor using an affinity resin containing anti-AFP antibody. While the low salt, 8S estrogen receptor did not interact with AFP antibody, the salt-dissociated 4S receptor form was adsorbed to the affinity resin. From these results, it was suggested that AFP is the uterine estrogen binding protein and that AFP associates with another cellular component to form an 8S complex which is resistant to anti-AFP antibody. This is an exciting proposal that could have extensive implications as to the evolutionary origin of these proteins and their functional diversity throughout development of the organism.

We have attempted to confirm these observations in the mouse using similar techniques. A partial adsorption of uterine estrogen receptor to an anti-AFP affinity resin was observed; however, a number of controlled experiments indicate that this binding was not to anti-AFP antibody but appeared to be a nonspecific interaction with the resin.

Materials and methods. [2,4,6,7-^{3}H]Estradiol-17β (91.3 Ci/mmol) was obtained from New England Nuclear. Unlabeled estradiol, ovalbumin, and activated charcoal were from Sigma; Sepharose-4B and dextran T-70 from Pharmacia, Uppsala, Sweden; and Biogel P-20, from Bio-Rad Labs.

Amniotic fluid was obtained from HA/ICR Mice by puncturing the amniotic sacs of animals on the 15–18th day of pregnancy. The fluid had a protein concentration of about 3 mg/ml and was stored at −20°.

Female HA/ICR mice (20–30 days old) were used as a source of estrogen receptor. The uteri were removed immediately after cervical dislocation, trimmed of fat and placed in saline at 4°. They were then transfered to TE buffer (10 m*M* Tris–HCl, 2 m*M* Na_2EDTA, 2 m*M* mercaptoethanol, pH 7.4) (5 uteri/ml) and homogenized using a Polytron PT10 homogenizer (Brinkman). The cytosol fraction was obtained by centrifugation for 1 hr at 150,000*g*. Solid KCl was then added to the cytosol to a concentration of 0.4 *M*.

Antisera to AFP and transferrin were prepared from mouse amniotic fluid (MAF) as follows. MAF was chromatographed on Biogel P-20 to remove low molecular weight components. The void volume was pooled and subjected to polyacrylamide electrophoresis at pH 9.5 in 5% gels (12). The gels were then sliced with a Gilson gel crusher and eluted with phosphate buffered saline, pH 7.2 using 1 mm slices. The locations of the AFP and transferrin in the eluted fractions were tested using specific antisera as well as antisera against anti-whole mouse serum. Fractions showing only AFP or transferrin by Ouchterlony were used to prepare antisera in rabbits. Mouse α-1 antitrypsin (α1AT) was prepared as previously described for the human (13). Anti-AFP showed a single line by

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Ouchterlony gel diffusion and immunoelectrophoresis with MAF and no reactions with normal adult mouse serum. Rabbit anti mouse α1AT and anti transferrin were monospecific as tested by gel diffusion and immunoelectrophoresis. The α-globulins of these antisera were precipitated with ammonium sulfate and linked to Sepharose-4B by cyanogen bromide (14).

Uterine cytosol was mixed with the immunoadsorbant resins in the proportion 0.8 ml cytosol to 0.2 ml packed resin. The mixtures were incubated for 3 hr at 5° with gentle shaking. Similar incubations were performed using mouse amniotic fluid in place of uterine cytosol. The amniotic fluid was first diluted 200-fold in TE buffer plus 0.4 *M* KCl and ovalbumin, 1 mg/ml. One milliliter portions were mixed with 0.2 ml packed resin and incubated for 3 hr at 5° with gentle shaking.

After incubation, the samples were centrifuged (2 min, 800*g*) to remove resin and the supernatant fluids were tested for [^{3}H]-estradiol binding. This was accomplished by mixing duplicate aliquots (0.3 ml of cytosol samples or 0.05 ml of amniotic fluid samples) with TE buffer + 0.4 *M* KCl to a final volume of 0.5 ml. [^{3}H]Estradiol (in TE buffer) was also added to a final concentration of 1×10^{-9} *M*. Additional assay tubes were included which contained a 400-fold excess of unlabeled estradiol for background determinations. The assay tubes were incubated in ice bath for 16 hr. The bound and unbound hormone were then separated by charcoal treatment (15, 16). Five-tenth milliliter of charcoal suspension (0.25% charcoal, 0.025% dextran in TE buffer) was added to each assay tube and mixed rapidly. The samples containing amniotic fluid were centrifuged immediately (1000*g*, 5 min) to minimize dissociation of the estradiol-AFP complex (dissociation was estimated to be less than 20%). The samples containing uterine fluid were incubated with charcoal for 10 min at 4°. The estradiol receptor complex does not dissociate significantly during this treatment (15). After centrifugation, the supernatants containing the bound hormone were decanted into scintillation counting vials, and 5 ml of scintillation fluid (1000 ml toluene plus 42 ml Scintiprep #1, Fisher) were added to each vial. After waiting 3 hr to extract the [^{3}H]estradiol into the toluene phase, the radioactivity was measured with 48% efficiency.

Results. In the studies of Uriel *et al.*, estradiol binding was determined by sedimenting the complex on sucrose gradients (11). We used this technique in our initial experiments with only limited success. There was some loss of estradiol binding when samples were treated with the anti-AFP antibody resin. However, the results were variable and the loss of binding was not complete. In addition, the sucrose gradient analysis is complicated by the fact that, under the ionic conditions of these experiments (0.4 *M* KCl), both the receptor and AFP sediment in the 4S region. Therefore, we decided to use the charcoal adsorption assay which we felt was more quantitative and would readily distinguish receptor binding from binding which was either non-specific or to components with lesser affinity.

Although it is possible to measure accurately estrogen binding to mouse AFP using the charcoal techniques, the charcoal incubation must be brief and precisely timed because of the rapid rate of estradiol dissociation from AFP. This property was used to easily distinguish hormone binding to AFP and to the estrogen receptor. The estradiol-AFP complex dissociates rapidly and is 90% dissociated after 10 min of charcoal incubation (Fig. 1). The half-time for hormone dissociation is between 2 and 3 min. This is somewhat faster than the dissociation rate of the estradiol-AFP complex in the rat (16). That the observed binding was actually to AFP was verified in separate experiments which showed the affinity and specificity of binding to be very consistent with previous studies (1–6). In addition, the binding was selectively removed by antibody to mouse AFP (see below).

Contrary to the results with AFP, the binding in uterine cytosol is almost completely stable during a 10-min period of charcoal

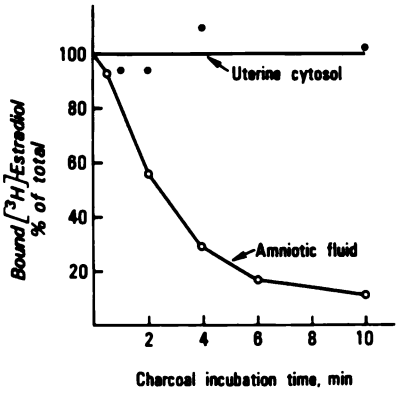


FIG. 1. The effect of charcoal treatment on the binding of [^{3}H]estradiol to receptors in uterine cytosol or to AFP from amniotic fluid. A series of assay tubes (in duplicate) were prepared containing either 0.3 ml cytosol or 0.05 ml amniotic fluid (diluted 200-fold) in a final volume of 0.5 ml. The samples were incubated with 1 × 10^{-9} *M* [^{3}H]estradiol for 16 hr and then treated with charcoal (see Methods and Materials) for various time periods. The bound hormone in the supernatant was measured after centrifugation. The value for 100% binding in the AFP assay was estimated by graphing CPM bound on a log scale versus time and extrapolating to zero time. These values were 2600 cpm for AFP and 25,000 cpm for the uterine cytosol.

treatment (Fig. 1). This agrees with other reports which have shown the dissociation of estradiol-receptor complex to be extremely slow at 0–4° (15, 18). Therefore, the 10-min charcoal treatment would easily distinguish between binding to uterine receptor or to AFP.

Figure 2A illustrates the binding of [^{3}H]-estradiol in uterine cytosol after the cytosol had been treated with Sepharose containing anti-AFP antibody (see legend and Methods section for details). While there was some loss in binding after the treatment, this appeared to be nonspecific since comparable losses occurred after treatment with anti-α1AT antibody, ovalbumin linked to Sepharose, or with Sepharose alone. A much greater loss occurred after treatment with Sepharose containing anti-transferrin antibody. Since this column had been in use for 6 months or more, we subsequently prepared a second antitransferrin antibody resin and this did not show the same results (Fig. 2B) even though the immunoabsorbant was prepared from the same bleeding in both cases. This suggested that the first results may have also been nonspecific.

To verify the effectiveness of the anti-AFP antibody resin, the binding of [^{3}H]-estradiol to AFP from mouse amniotic fluid was tested before and after resin treatment (Fig. 3). The hormone binding was completely removed by the anti-AFP resin, but no effect was observed after treatment with the four control resins. Therefore, the experimental conditions were quite adequate for achieving the selective removal of AFP from solution. Both antitransferrin and the anti-α1AT completely removed their respective antigens from MAF as tested immunologically.

Several experiments of the type illustrated in Fig. 2 were performed and while variable losses of hormone binding occurred in these experiments, in no case was there a selective removal of receptor binding by the anti-AFP antibody resin.

Discussion. The present results do not support the idea of antigenic similarity between

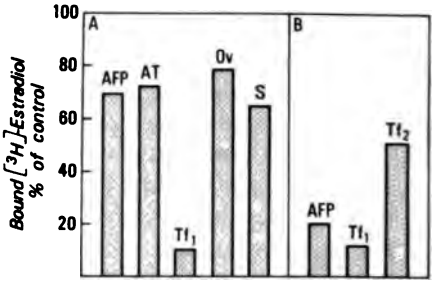
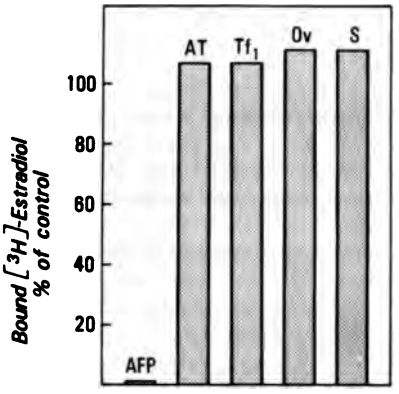


FIG. 2. The binding of [^{3}H]estradiol in uterine cytosol after treatment with Sepharose resins. Samples were incubated with the resins indicated for 3 hr at 5°. The resins were removed by centrifugation and the binding of [^{3}H]estradiol was measured in the remaining supernatant fractions. Values are expressed as percent of control. The control samples had no resin treatment. Experiment A: Samples of uterine cytosol were incubated with the following resins: anti-AFP antibody–Sepharose, AFP; anti-α1AT antibody–Sepharose, AT; antitransferin antibody–Sepharose, Tf_1, (first preparation); ovalbumin–Sepharose, Ov; untreated Sepharose, S. The control binding was 36,500 cpm and the background (plus 500-fold excess unlabeled estradiol) was 2000 cpm. Experiment B: This experiment was performed exactly as experiment A and represents a comparison of two preparations of antitransferin antibody–Sepharose, Tf_1, and Tf_2.



G. 3. The binding of [^{3}H]estradiol in amniotic fluid treatment with Sepharose resins. Samples of am- : fluid were treated with the indicated resins and stradiol binding was measured in the remaining natant fractions. The control value (without resin nent) was 27,000 cpm and the background was 1000

estrogen receptor and AFP. In several riments, we found that the estrogen re- or was adsorbed, in part, to resins of obilized AFP antibody. However, these lts were variable and the receptor adsorp- was never complete. In addition, this rption appeared to be rather nonspecific e a similar adsorption of receptor was rved using several other control resins h did not contain AFP antibody. These rvations are not surprising since several stigators have noted a tendency of estro- receptor to aggregate and to adsorb to ous surfaces (19–21).

he binding analysis used in the present y is different than that of Uriel *et al.* who l sucrose gradient centrifugation (11). vever, in our initial studies using sucrose lient centrifugation (data not shown), the lts were similar to those illustrated here the charcoal adsorption assay.

recent report by Radanyi *et al.* (17) also cated that rat AFP and the uterine estro- receptor were separate proteins. Their clusions were based on a comparison of binding specificities and dissociation rates hese components. Another preliminary rt by LaBarbera and Linkie (22) claimed ck of interaction between the rat uterine estrogen receptor and antibody to rat AFP in solution.

It is possible that antigenic similarities occur between AFP and estrogen receptor of the rat, but not the mouse. However, this seems very unlikely since the two species are closely related. Another possibility is that a small region of similarity exists between AFP and estrogen receptor. For example, some similarities may well exist at the steroid binding sites of the two molecules. If the common region represented an antigenic determinant in the system of Uriel *et al.* but not in our system, then our discrepant results could be explained. We have found that our antiserum does not mask the estrogen binding site of mouse AFP since the ability to bind steroid is not diminished after the interaction of AFP with antibody.

While the above possibilities exist, we feel that the simplest and most likely interpretation is that there really is no antigenic similarity between AFP and estrogen receptor. These proteins have very different physiochemical properties even though they both bind estrogen. Both the affinity and specificity of estrogen binding by AFP differ from receptor binding (11, 17, 23). The rate of dissociation of estradiol by AFP is very rapid in comparison to receptor binding. Also we have observed that unlike the estrogen receptor, AFP is a stable protein. Its binding activity is insensitive to sulfhydril agents such as *N*-ethylmaleimide and it can withstand incubations at elevated temperatures (e.g., 10 min, 60°). Finally, estrogen receptors have very similar properties among all mammalian species that have been studied (24), whereas estrogen binding by AFP shows species differences and bovine and human AFP have little or no affinity for estrogens (7; N. Calvanico, D. Toft, and T. Tomasi, unpublished).

Summary. This study was an attempt to identify antigenic similarity between two estrogen binding proteins in the mouse; the uterine estrogen receptor and α-fetoprotein. The adsorption of these proteins onto an affinity resin containing immobilized anti-

body to α-fetoprotein was tested. Alpha-fetoprotein could be selectively bound to this resin. On the other hand, estrogen receptor was only partially bound to the resin and this appeared to represent a nonspecific adsorption to the resin when tested against several control resins. Therefore, no antigenic similarity between these two proteins was observed.

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ranulocyte Sepcration by Modified Centrifugal Elutriation System (40105)

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nt laboratory methods for the sepa- f granulocytes are, to a greater or egree, damaging to the cells. The most frequently used are isopyknic gradient centrifugation and separa- the reversible adhesion to nylon or faces. The harmful effects of these res have been reported by a number igators (1, 2). These methods subject to the physical trauma of repeated gation and packing, are laborious and ow granulocyte yields. Clearly, there for a better technique for separation locytes.

w process, CE,[1] has been recently ed and successfully used for the iso- f a number of different cell types i this process sedimentation of cells ntrifugal force is counteracted by the g force of liquid flow. The cell sepa- sults when a portion of the cell pop- nas a sedimentation rate slower than velocity and is washed out from the on chamber, while more rapidly sed- g cells remain suspended in the cham- e retained cells are eluted either by ng the flow rate or by decreasing the eed. During this separation process, ting and prolonged contact with for- faces are avoided. This method allows of any physiologically suitable me- roduces a high yield of cell fractions nigh degree of purification, and re- relatively short processing time.

attempt to utilize CE for separation ulocytes from whole human blood, n *et al.* (8) reported that it was nec- rst to enrich the leukocyte population tran-sedimentation of blood before hocyte-free granulocyte preparation e obtained by elutriation. Because l advantages of CE promise to elim- inate many shortcomings of the previous techniques, we have investigated the feasibility of employing CE for the one-step separation of granulocytes from whole human blood. This investigation and our earlier studies (9, 10) show that, with proper modification, the CE process is most effective for separation of granulocytes from whole human blood without the need for the leukocyte enrichment step.

Materials and Methods. Blood collection. Blood (5 ml) was collected by venipuncture from healthy volunteers. It was immediately diluted tenfold with HBSS[2] which effectively prevents blood clotting for several hours. This method was adapted from the procedure reported by Chodirker *et al.* (11) in which blood clotting was prevented by twentyfold dilution with saline containing 0.1% bovine serum. For comparison, blood was anticoagulated by heparin (10 U/ml), and then used either undiluted or diluted tenfold.

Cell separation system. The Beckman JE-6 elutriator rotor was used in the Beckman J-21 preparative centrifuge. In order to achieve maximal cell yield and minimal dilution of the purified granulocytes, the flow system of the Beckman elutriator has been substantially modified by the senior author. The main modification is in providing the means for reversing the direction of fluid flow through the separation chamber in the rotor while the system is in operation. This is accomplished by using a compound valve which reverses both the direction of fluid flow and at the same time changes the exit line from waste to sample collection. The diagramatic representation of this new flow system is shown in Fig. 1. Changing the centripetally oriented flow after the completion of elutriation to the centrifugal direction aligns the action of both the fluid drag and the centrifugal force. This facilitates the evacuation of granulocytes from the separation chamber. As a result, the

ork was supported by the NIH Grant No. CA

fers to centrifugal elutriation.

[2] HBSS refers to Hanks' balanced salt solution.

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concentrated suspension of granulocytes can be collected in aliquots of small volume (5 to 8 ml). In contrast, elution of cells by the original Beckman system yields collected cells in aliquots of much larger volume (150 ml). Once the cells are eluted, the system is ready for the next run, so that many blood samples can be successively processed. By reversing back and forth the direction of flow, it is possible to unclog the separation chamber from cell pellet while the system is in operation should cell packing occur. Another modification of the Beckman system is that the mixing chamber was replaced with the inverted sample bottle as shown in Fig. 1. During the loading step the diluted blood is displaced by air from the sample bottle and driven along the fluid line into the rotor, and into the separation chamber.

CE procedure. The CE procedure starts by purging air from the assembled flow system which is facilitated by reversing the direction of flow several times. The rotor speed is then set and maintained at 2500 rpm throughout the entire operation. Rotor speeds lower or higher than 2500 rpm have been found less suitable, as will be discussed later. During the loading of the diluted blood, the rate of the fluid flow is maintained at 11 ml/min. Following completion of the blood loading the flow rate is increased to 14 ml/min. After approximately 15 min, the separation chamber begins to clear at its centripetal side while remaining turbid at the other side (Fig. 2). The visible turbid zone upon microscopic examination was found to consist predominantly of granulocytes mixed with some red cells. To speed up the purification proce flow rate is increased until the bound the turbid zone is displaced to the top wide side of the chamber. Based on a of 68 experiments, optimal flow rate fc aration of granulocytes was found to from 14 to 35 ml/min, depending upc individual donor. The flow rates from (of a total of 68 runs were in the ran 14–25 ml/min. Blood specimens used f study were obtained from eight subjec correlation was found between the indi subjects and the flow rate variations measured on different days. Howeve flow rates were highly reproducible fro to run, when measured on the same da blood from the same subject. Followi min of elutriation the granulocyte suspe becomes virtually free of red cells and phocytes. The concentrated granulocyt then collected by reversing the directi the flow through the separation chambe entire system was maintained at room perature throughout the operation.

Dextran–sedimentation. For compa leukocytes were collected by the dextran imentation technique (12). Five ml of h rinized (10 U/ml) blood were mixed equal volume of 3% Dextran 5000 (Pha cia, Uppsala, Sweden) in saline and all to sediment at room temperature for 45 The leukocyte-rich supernatant was drawn, centrifuged at 80*g* for 6 min, washed twice with HBSS. The numb granulocytes per ml was determined by a hemacytometer.

Evaluation of granulocyte function.

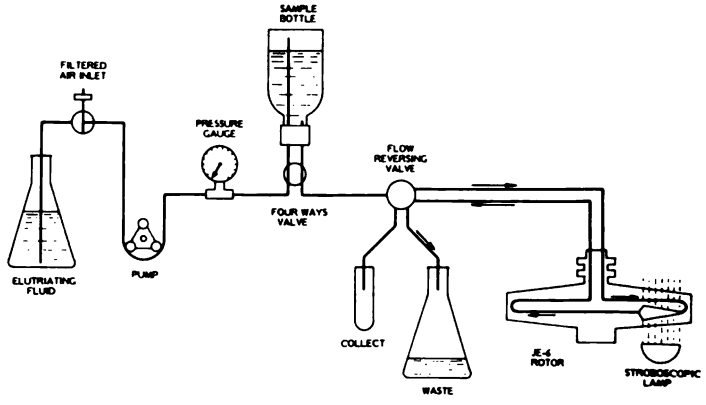

FIG. 1. Flow diagram of the modified centrifugal elutriation system.

FIG. 2. Beckman's separation chamber in the process of elutriation, photographed with the strobe light. Th bottom two thirds of the chamber contains a suspension of purified granulocytes separated from 5 ml of whol blood. Clotting was prevented by tenfold dilution of the blood. 2 × enlargement.

function of granulocytes isolated by CE at different rotor speeds (2500, 3000, 4000 and 5000 rpm) was evaluated for their bactericidal and phagocytic abilities and their membrane integrity. The ability of granulocytes to kill *S. aureus* was compared to that of dextran-sedimented leukocytes from the same donor. Each sample tube contained 0.1 ml cell suspension (1×10^6 granulocytes), 0.1 ml AB+ serum, 0.025 ml bacterial suspension (1×10^6 bacteria), and 0.775 ml HBSS. Samples wer incubated at 37° for 120 min. Then, the were plated on agar, incubated at 37° for 2 hr and analyzed for bacterial growth. Resul were expressed as the number of bacteri surviving after 120 min of incubation wit granulocytes (13). Phagocytic function an membrane integrity of granulocytes were ar alyzed by our method (14) involving the com bined use of fluorescein diacetate, ethidiu

bromide, and zymosan particles. This method allows easy distinction between phagocytizing cells, nonphagocytizing cells, and dead cells.

Results. Granulocyte separation. The results of this study on the use of the modified CE system for separation of granulocytes from blood are based on daily experimental runs conducted over a period of two years. The systematic study of different flow rates during the loading of whole blood revealed that the flow rate must initially be kept low (11 ml/min). To obtain maximal granulocytes yield the volume of loaded blood should not exceed 5 ml. Loading larger volume of whole blood than 5 ml results in considerable loss in recovery of granulocytes. Apparently, this is due to overcrowding of cells in the chamber. When tenfold diluted blood is used then a total of 10 ml whole blood can be loaded without significantly reducing the granulocyte yield. The initial dilution allows red blood cells to be continuously eluted from the separation chamber during loading which relieves the overcrowding and improves the condition for retention of granulocytes.

The entire procedure for separating granulocytes from 5 ml of whole blood takes between 30 and 35 min at 2500 rpm, depending upon donor. The collected granulocyte preparation contains approximately 2:1 ratio of erythrocytes to granulocytes. At this point very few lymphocytes are present. From 5 ml of whole blood, the number of lymphocytes and granulocytes from five separate experiments averaged $2.0 \times 10^5 \pm 1.9 \times 10^5$ and $137 \times 10^5 \pm 46 \times 10^5$ cells respectively. The few lymphocytes present constituted only about 1.7% of the total. A complete removal of both lymphocytes and erythrocytes can be achieved after about 90 min of elutriation. Larger blood samples of up to 30 ml after tenfold dilution with HBSS can also be processed with fair yields of granulocytes.

Granulocyte yield. Yields of granulocytes separated by this procedure from diluted blood can be as high as 100%. Typically, the yield approaches 90%, with an average value of 87%. Lymphocytes present in the separated granulocyte samples gave an average value of $1.2 \pm 1.1\%$. The calculation of granulocyte yield was based on differential WBC counts in the samples of whole blood before elutriation and in the collected granulocytes after elutriation.

Cell evaluation. Granulocytes isolated by CE at different rotor speeds and different flow rates were evaluated for their bactericidal function, phagocytic activity and membrane integrity. The results of these tests are presented in Table I. It is evident from these data that, at a rotor speed of 2500 rpm and a flow rate of 21 ml/min, the ability of granulocytes to kill *S. aureus* is comparable to that of Dextran-sedimented control cells. However, with the increase of rotor speed up to 5000 rpm and higher flow rates up to 80 ml/min, there is a significant decrease in the bactericidal ability of granulocytes. Phagocytic function and membrane integrity tests show an apparent decrease in phagocytic capacity up to 4000 rpm while membrane integrity did not display significant changes with increase in rotor speeds. Phagocytic function of granulocytes obtained at 5000 rpm appears to approach the level observed with cells elutriated at 2500 rpm. At this high rotor speed the elutriation rate is very high (80 ml/min), therefore, a much shorter time (10 min) is required to separate granulocytes almost free of lymphocytes and red cells than that at 2500 rpm (35 min). The shorter processing time could be one of the reasons for the recovery of phagocytic function of gran-

TABLE I. EFFECT OF ROTOR SPEED AND ELUTRIATION RATE ON FUNCTIONS OF GRANULOCYTES

Rotor speed rpm	Elutriation rate ml/min	% Phagocytizing cells[a]	% Nonphagocytizing cells	% Dead cells	% Killing of *S. aureus*[c]
2500	21	56 ± 6[b]	41 ± 4	3 ± 0.5	92 ± 3.1
3000	38	38 ± 4	44 ± 5	18 ± 3	84 ± 1.3
4000	48	28 ± 17	60 ± 19	12 ± 2	80 ± 1.6
5000	80	50 ± 16	41 ± 16	9 ± 0.7	62 ± 5.5

[a] As control experiments for phagocytic function, Dextran-sedimented leukocytes were used and the numbers of phagocytizing cells were between 50 and 63%.

[b] Standard deviations.

[c] The percent killing of *S. aureus* by Dextran-sedimented leukocytes was 91 ± 4.4

:s (Table 1). Rotor speed below 2500 quires longer than 35 min of elutriation id therefore is less desirable.

nulocytes isolated by this procedure blood diluted tenfold with anticot have a lesser tendency to clump, larly upon incubation at 37°, than solated from heparinized blood. Also ave less tendency to clump than do n-sedimented cells.

ussion. The modifications of the Beck- entrifugal elutriator concern mainly s flow system. By adding new means ersing the direction of flow through aration chamber and by making dif- provisions for loading cell samples, this becomes more effective for the sepa- of granulocytes than was the original as described by McEwen (8). The ages resulting from these modifica- re that with this system one can: (a) te granulocytes from whole human in one-step procedure; (b) collect con- ed cells in small volume; (c) obtain 100% yield of purified granulocytes;) unclog the separation chamber with- pping the rotor, should cell packing by simply reversing direction of flow. on the evaluation of the granulocytes' cidal and phagocytic function, and on embrane integrity, it appears that our cedure is less traumatizing to the cells re the other available methods. It is to perform, requires a relatively short nd is suitable for serial processing of blood samples. In addition, granulo- an be separated without the use of gulants. Therefore, this procedure ap- o have a greater potential than other le methods for obtaining granulocytes least damaged condition for a variety cal tests, such as chemotaxis, migration ion, NBT reduction, etc., as well as for of scientific studies on the biochemical of granulocytes. Although some of linical tests do not require pure gran- s, the use of intact and purified cells make the tests more efficient, specific sitive.

order in which the blood cell types are uted in the separation chamber during tion appears, paradoxically, to be the of that commonly observed during the centrifugation of blood. During elutriation, granulocytes occupy the centrifugal side of the chamber while red cells and lymphocytes the centripetal side. It is interesting to note that Maupin (15) showed that when blood is entensively diluted with normal saline and then centrifuged, granulocytes are sedimented first, followed by lymphocytes and red cells. Since the CE process also involves extensive dilution of blood, this may be a reason for the analogous distribution of the blood cells within the elutriation chamber.

Of special interest is the observed reproducibility in the flow rates required to maintain granulocytes at a specified level within the separation chamber when measured on the same day with the blood from the same donor. This reproducibility suggests that flow rate variations are not due to the experimental error, but rather due to actual variations in the granulocyte volume or bouyant density. Thus, CE may offer a new way for measuring in bulk this important cell parameter.

Summary. The Beckman centrifugal elutriation system has been modified for a one-step separation of granulocytes from whole human blood. A special valve for reversing the direction of flow permits the collection of concentrated granulocytes in small volumes and allows unclogging of the separation chamber should cell packing occur during centrifugal elutriation. Blood diluted tenfold prevents coagulation, reduces the cell trauma and makes separation more efficient. Maintenance of the precise flow rates by visual monitoring of the granulocyte level in the chamber during elutriation, when the chamber begins to clear of red blood cells, is essential for optimal separation. Granulocytes isolated at rotor speed of 2500 rpm are indistinguishable in their membrane integrity, bactericidal and phagocytic abilities from those collected by Dextran-sedimentation. Cell yields close to 100%, high purty, minimal cell traumatization, and short processing time make this technique highly effective in collecting granulocytes for clinical and laboratory studies.

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actin Secretion from Clonal Pituitary Cells following Incubation with Estradiol, Progesterone, Thyrotrophin Releasing Hormone and Dopamine (40106)

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ro studies utilizing anterior pituitary ave been employed for a number of study the physiologic role of specific on one or more of the hypophyseal es. One of the cells that remains via- secretes its hormonal product in cop- ounts when placed into an artificial ment is the prolactin cell (1, 2). A of compounds have been used to ne their effect on prolactin (PRL) se- *in vitro*. Some of those which have own to be stimulatory are estrogen stosterone (5), thyrotrophin releasing e (TRH) (5–7), cyclic AMP (8), and (9, 10), while inhibition has been ob- with dopamine hydrochloride (DA), 2), apomorphine (8), progesterone (P) the ergot alkaloids (8, 11, 13).

al investigators have obtained en- ell fractions of rat anterior pituitary m normal hypophyses (14–16). Hy- l coworkers have successfully isolated ich fraction of cells from adult female tary glands by velocity sedimentation gravity (17) and have used these cells ine the kinetics of PRL secretion (18). of pituitary cells have been established mura *et al.* (19) and Tashjian *et al.* m a rat pituitary tumor. These cells st reported to produce either adreno- rophin (ACTH) or growth hormone 9). In a later study several of the GH- g strains were also shown to secrete l). An attempt was made by Steinber- *l.* (22) to establish gonadotrophin se- cell clones from normal rat pituitary They found that a majority of their ells secreted neither gonadotrophic e and that gonadotrophs could not be intained in culture. Recently, Ishi- *al.* (23) reported the establishment thke's pouch tissue derived from nor- fetuses of 65 clonal strains of pituitary ich produce PRL, GH and/or ACTH. The following investigation utilized one of these clones (2B8) to examine the direct effects of several compounds on PRL secretion.

Materials and methods. A clone of pituitary PRL cells, designated 2B8, was derived from the epithelium of Rathke's pouch as previously described (23). The cells were placed into Falcon culture dishes (60 × 15 mm, #3002) containing growth medium which consisted of 85% Ham's F10 medium and 15% virus screened fetal calf serum (Gibco Biological Supplies), 50 μg/ml streptomycin, 50 U/ml penicillin and 0.5 μg/ml fungizone. Each dish contained a total volume of 4 ml of growth medium and approximately 1×10^5 cells. After seeding, the dishes remained at room temperature for 2 hr and were then transferred to a humidified CO_2 incubator, and maintained at 37° with a gas mixture of 5% CO_2, 5% O_2, and 90% air for 10 days. Following this, the medium was discarded from each dish and replaced with fresh growth medium containing 10^{-8} *M* E_2 [estradiol sodium sulfate (Steraloids, Inc.)],[1] 2×10^{-9} *M* TRH (Beckman, Inc.), or a mixture of both 10^{-8} *M* E_2 and 2×10^{-9} *M* TRH. After 3 days, the medium was again discarded and replaced with fresh growth medium containing the aforementioned concentrations of E_2, TRH, or E_2 and TRH alone or in combination with 10^{-6} *M* DA (Sigma Chemical Corp.). After an additional 6 hr, the medium was collected and stored frozen for subsequent quantitation of PRL content by radioimmunoassay. The 2B8 cells were also cultured using the same protocol (10 days growth medium-3 days growth medium containing secretagogue-6 hr growth medium containing secretagogue or inhibitor) with

[1] The estradiol and progesterone were initially dissolved in absolute alcohol and then put into the growth medium. The final concentration of alcohol in the steroid-containing medium was 0.5%.

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varying concentrations of E_2 or P [4-pregnen-3,20 dione (Mochida Pharmaceuticals Co. Ltd.)][1] ranging from 10^{-11} *M* to 10^{-6} *M*, 10^{-8} *M* E_2 plus 10^{-6} *M* P, or with 10^{-9} *M* to 10^{-5} *M* of DA. In all studies, 10 dishes were cultured in triplicate for each dose level of secretagogue or inhibitor employed. In addition, two types of control cultures were routinely performed using the aforementioned 10 day-3 day-6 hr protocol. In the first, a series of dishes containing only growth medium and no cells were utilized ("medium only" control), and in the second, the dishes contained growth medium and 2B8 cells only ("cells only" control). After each experiment, the cells were stained with 0.1% trypan blue and counted using a hemocytometer.

The PRL concentration in the media was measured by radioimmunoassay using a homologous rat PRL assay system employing reagents supplied in kits by NIAMDD. The rat PRL was iodinated using a slight modification of the lactoperoxidase procedure as described by Aubert *et al.* (24). The inter- and intra-assay coefficients of variation were 8.2% and 6.7%, respectively. All values are expressed in terms of NIAMDD rat PRL-RP-1. The data were statistically analyzed by analysis of variance and by the *t* test for comparison of differences between means.

Results. The amount of PRL released into the media by the 2B8 cells incubated with varying concentrations of E_2 and P is shown in Fig. 1. The concentration of PRL in the dishes containing 10^{-11} *M* E_2 was essentially the same as the "cells only" control. In contrast, there was a dose-related increase in PRL levels in the media from cells incubated with 10^{-10} *M*–10^{-8} *M* E_2, with the maximum amount of PRL secretion occurring with 10^{-8} *M* E_2. When 10^{-7} *M* and 10^{-6} *M* E_2 were utilized, PRL release declined to levels which closely approximated values recorded in the "cells only" controls. In this study as well as in the subsequent experiments, PRL was not detectable in the "medium only" control. A dose-related decrease in PRL secretion was observed following the incubation of the 2B8 cells with concentrations of P ranging from 10^{-11} *M* to 10^{-6} *M*. Moreover, an inhibition in the amount of PRL secreted by the cells was recorded when 10^{-8} *M* E_2 and 10^{-6} *M* P were simultaneously added to the culture me-

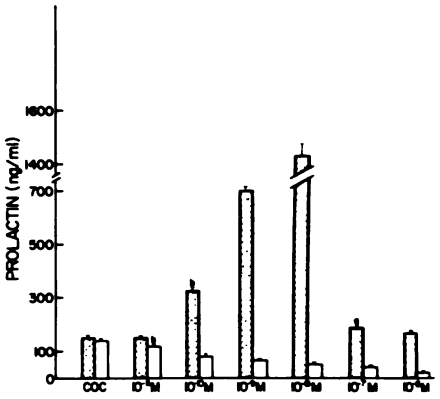

FIG. 1. Prolactin concentration (mean ± SE ture media from 2B8 cells incubated with E_2 (bars) or P (open bars), from 10^{-11}*M* to 10^{-6} *M*. *M* E_2 and 10^{-6} *M* P (cross hatched bar). a = n < 0.001 as compared to "cells only" control (C(

dium. This, however, was not as great decrease noted with P alone. Besides af PRL production by the 2B8 cells, E_2 also induced changes in cell proliferati stimulated a significant increase in cel ber at all concentrations examined whi dose-related. This increase ranged fror in the group incubated with the lowe centration of E_2 to 42.3% in the grou bated with 10^{-6} *M*. The dose of E_2 caused the maximum release of PRI stimulated a 19.3% increase in cell nu In contrast, P inhibited cell proliferatio effect was statistically significant onl concentrations of 10^{-8} *M*–10^{-6} *M*. No (in cell number was observed when tl steroids were incubated together.

There was nearly a twofold rise i release from cells cultured with 10^{-9} (Fig. 2). However, with higher concent of DA, a decline in PRL release was ob which was significantly lower than the only" control at dosages of 10^{-7} *M*–1(The total cell number in dishes cont DA-supplemented media was the sa that present in the "cells only" control

The results of the study in which E *M*), TRH (2×10^{-9} *M*) and DA (1(were incubated alone or in combinati shown in Fig. 3. Alone, E_2 and TRH edly stimulated PRL release. There statistical difference in the amount c secreted by the cells incubated in the pi

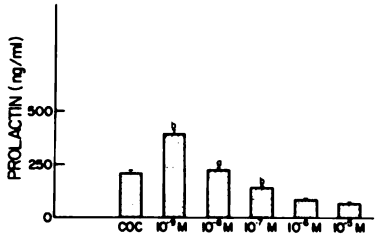

FIG. 2. Dose-related secretion pattern of PRL (mean ± SE) by 2B8 cells following incubation with 10^{-9} *M* to 10^{-5} *M* DA. a = ns, b = $P < 0.001$ as compared to "cells only" control (COC).

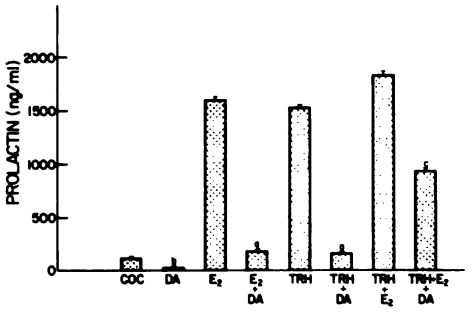

FIG. 3. Effect of 10^{-6} *M* DA, 10^{-8} *M* E_2 and 2×10^{-9} *M* TRH alone or in combination with each other on PRL secretion (mean ± SE) from 2B8 cells. a = ns, b = $P < 0.001$ as compared to "cells only" control (COC).

of E_2 as compared to the response from TRH. When the cells were cultured with a combination of E_2 and TRH, significantly more hormone was released ($P < 0.001$) than when each was incubated alone, albeit this effect was not additive. Simultaneous incubation of E_2 and DA or TRH and DA resulted in a marked decrease in PRL release to levels which were similar to that observed in the "cells only" control. The inhibitory effect of DA on the combination of TRH and E_2, however, was not as great. TRH and E_2 alone, in combination with each other or with DA significantly increased cell proliferation. DA alone had no effect.

Discussion. The present investigation demonstrates the first use of a normal diploid clone of pituitary cells for the study of the regulatory mechanisms that control PRL secretion. The 2B8 clone has previously been shown by radioimmunoassay to secrete only PRL (23). The only other viable clones of pituitary cells that have been extensively used experimentally are those developed by Tashjian and coworkers from rat pituitary tumors (19, 20). While these were initially found to produce more than one hormone (19–21), subsequent studies with subclones derived from these strains have shown that certain strains secrete only GH or PRL (21, 25).

In our experiments, the 2B8 cells have been shown to be responsive to E_2, P and TRH much like the GH tumor cells. In comparing our observations with those of Haug and Gautvik (5), it can be noted that the tumor cells initially responded to tenfold less E_2 than the 2B8 cells; however, both cell strains produced a maximum amount of PRL with 10^{-8} *M* E_2. At higher concentrations (up to 10^{-6} *M*), PRL secretion by the tumor cells plateaued, while that of the 2B8 cells fell precipitously. Observations similar to ours with varying doses of E_2 have been obtained *in vitro* using organ cultures of anterior pituitary tissue (4) and in ovariectomized rats given dosages of 0.1–500 μg estradiol benzoate (26). At all dose levels of P tested, there was significantly less PRL released from 2B8 cells than occurred in the "cells only" control cultures. In contrast, Haug and Gautvik (5) observed significant changes in the amount of PRL released by the tumor cells only with 10^{-8} *M*–10^{-6} *M* P. Although slightly less PRL was seen in the media containing 10^{-9} *M* P, they reported no significant inhibition with doses of 10^{-11} *M*–10^{-9} *M* P. When the tumor (5) or the 2B8 cells were incubated simultaneously with 10^{-8} *M* E_2 and 10^{-6} *M* P, less PRL was secreted than with E_2 alone. The suppressive effect of P on E_2 was greater on the 2B8 cells than on the GH tumor cell line. In the presence of the two steroids, the tumor cells produced 124% more PRL than their control cultures (5) while the 2B8 cells secreted 28% less than the "cells only" controls. An inhibition of the stimulatory effect of E_2 by P has also been observed *in vivo* following the simultaneous administration of 1 μg E_2 and 0.5, 1 or 4 mg P to ovariectomized rats (26). Both the 2B8 and the tumor cells were stimulated by TRH (Fig. 3; 5, 27). When the 2B8 cells were incubated with TRH and E_2, a 19% increase in PRL secretion was recorded over that obtained with TRH alone. In contrast, the GH_3 cells produced more than twice as much PRL under similar conditions (5).

The discrepancy in the two studies may be due in part to the difference in the amount of TRH employed, which in our experiment was 100 times less than that used by Haug and Gautvik (5); both investigations utilized the same concentration of E_2. The stimulation of PRL secretion by either TRH or E_2 was suppressed to control levels by DA, while simultaneous incubation of the 2B8 cells with TRH, E_2 and DA reduced PRL levels by about 50%. These observations are consistent with those of Hill-Samli and MacLeod (7).

Proliferation of the 2B8 cells was stimulated by TRH and E_2, inhibited by P and not affected by DA. There was no correlation between cell proliferation and PRL release or inhibition. Cell number was at its maximum with the dose of E_2 that failed to stimulate PRL release. Incubation of the cells with TRH or E_2 lead to a 15-fold increase in PRL release but only a 19–20% increase in cell number. DA inhibited hormone release but had no effect on cell proliferation. The degree of inhibition of PRL release by P was much greater than the extent to which cell number was reduced. In their studies with the GH_3 tumor cells, Haug and Gautvik (5) found no change in cell growth (as determined through quantitation of cell protein) with either E_2 or P. They speculated that either the cells had lost the ability to proliferate under the influence of E_2 or that the factor(s) responsible for cell growth were of hypothalamic origin. In light of our findings, the latter hypothesis seems unlikely.

Heretofore, the only reported investigation in which stimulation of PRL release was observed with DA was that of Malarkey *et al.* (28). They found an increase in PRL release by GH tumor cells using 4.5×10^{-5} *M* DA. A similar phenomenon was consistently observed from the 2B8 cells with 10^{-9} *M* DA. Since concentrations of DA in the hypophyseal portal blood have been reported to be in excess of 10^{-9} *M* (29), the significance of our observations remain unclear. The secretory pattern of the 2B8 cells was unaffected by 10^{-8} *M* DA. Likewise, no change in PRL release was observed following incubation of hemipituitary glands with approximately 1.2×10^{-8} *M* DA (30). Other investigators (7, 8, 12, 17) including ourselves who have employed DA at concentrations which more closely approximate those values found in the hypophyseal portal blood (29) have obtained a marked inhibition of PRL secretion. From these reports one would assume that DA acts to inhibit the secretion of PRL from the anterior pituitary gland. However, this premise has recently been challenged by Neill *et al.* (31) who failed to obtain a significant correlation between peripheral PRL levels and DA concentration in the portal blood during the estrous cycle. This then raises the question of what the physiological role of DA is in the regulation of PRL secretion.

Summary. Prolactin (PRL) release was assessed using clonal cells from the 2B8 strain grown in the presence of estradiol (sodium sulfate) (E_2), progesterone (P), thyrotrophin-releasing hormone (TRH) and/or dopamine hydrochloride (DA). Using concentrations of E_2 ranging from 10^{-11} *M* to 10^{-6} *M*, it was found that a significant amount of PRL was released with doses of 10^{-10} *M* to 10^{-8} *M*. No effect was observed with either higher or lower concentrations of the steroid. Approximately the same amount of PRL was released from pituitary cells incubated with 2×10^{-9} *M* TRH as was noted with 10^{-8} *M* E_2. When these two secretagogues were combined, significantly more PRL was present in the medium than when either was employed alone. Prolactin release was significantly inhibited from cells cultured with 10^{-7} *M*–10^{-5} *M* DA; lower concentrations were either ineffective or were found to stimulate PRL secretion. In combination with 10^{-8} *M* E_2 or 2×10^{-9} *M* TRH, 10^{-6} *M* DA significantly reduced the stimulatory effect of either of these two compounds. However, when E_2 and TRH were incubtaed together in the presence of DA, less of an inhibition of PRL was noted than when DA was combined with either E_2 or TRH. Inhibition of PRL release also occurred when the cells were incubated with 10^{-11} *M*–10^{-6} *M* P. In addition, the stimulatory effect of 10^{-8} *M* E_2 was found to be suppressed by 10^{-6} *M* P. The results indicate that the 2B8 clonal strain is sensitive to agents known to effect PRL secretion *in vivo*.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 610–614 (1978)

Pulmonary Vascular Resistance and Right Ventricular Function in Canine Endotc Shock[1] (40107)

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The primary lethal factor in endotoxin shock has been thought to be inadequate venous return in the dog, which was ascribed to splanchnic pooling (1). In our studies of splanchnic pooling in canine endotoxin shock (2, 3), we found that the portal vein constricted early and remained constricted until death; the liver trapped blood early, but had returned to the control size by thirty minutes after endotoxin, whereas cardiac output and aortic pressure remained below 60% of the control levels.

In our 1970 study in the dog (2), we obtained arterial blood samples for lactate, pH, and gases at hourly intervals. There was a progressive increase in lactate and a progressive decrease in pH, and a corresponding decrease in PCO_2, except for the last sample before death which showed an increase in CO_2. Similarly, there were no consistent changes in PO_2 except for an abrupt but mild drop in the last sample. It seemed safe to conclude from the trends of lactate and gases that circulatory function was compromised rather than pulmonary function in endotoxin shock.

The same center that proposed splanchnic pooling as critical in the pathogenesis of shock (1) was also the first to report the response of the pulmonary vasculature to endotoxin (4). Kuida and colleagues found an average 43% increase in pulmonary arterial resistance. They discounted the pulmonary vascular changes as a major factor in producing fatal shock in dogs; they attributed the lethal outcome to an unchanged peripheral resistance (5) in the face of a cardiac output reduced by splanchnic pooling (the cat, however, they accepted the p nary vascular changes as primary, lead "gross pulmonary edema, right heart f and sudden death." Similarly, Halmag coworkers (6) found that the total pulm vascular resistance increased threefo sheep, and this increase preceded the cardiac output. Halmagyi explicitly bl acute right ventricular failure for the output. An editorial in LANCET ev ferred to the phenomenon in sheep a pulmonale in endotoxin shock." The physiology in sheep was distinguished that in the dog because the cardiac c could be restored by transfusion in the (7), from which they concluded that i quate circulating volume was the centr order, in the dog.

At the time of Halmagyi's report in (6), most centers held that myocardial f did not play a significant part in the circulatory collapse (8, 9). More recentl fer and his colleagues (10), reported ocardial depressant factor (MDF) whic curs later in endotoxin shock, and Hin and co-workers also reported left ventr depression in the dog. Myocardial depr in the presence of increased pulmonar cular resistance, resulting in even mod increases in right ventricular afterload, produce acute right ventricular failure therefore have examined the pulmonar systemic vascular resistances during toxin shock in the dog, the right ventr performance, and the ability of dextra fusions to restore hemodynamic conditic normal.

Material and methods. Twelve dogs o selected sex and breed were used, wei 18–24 kg. They were premedicated with phine (2 mg/kg im), and anesthetized sodium pentobarbital (15 mg/kg iv).

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ventricular pressure was obtained by retrograde catheterization from the carotid artery, and the aorta from the femoral artery. Cardiac output was determined by dye-dilution with indocyanine green; computation was performed on a PDP 11/45. A Swan–Ganz balloon-tipped catheter was advanced from the femoral vein to the pulmonary artery. A second venous catheter was advanced to the right atrium. After control recordings were made, an LD_{100} dose of endotoxin (5 mg/kg) from *E. coli* (Westphal extraction method, Difco Laboratories) was injected into the right atrium. Recordings were made at 5, 15, 30, and 60 min, and at hourly intervals for 4 hr. The data were reduced manually; the cardiac output, mean aortic pressure, mean pulmonary artery pressure, and vascular resistances were normalized for each animal, with the control value as 100%. For the low pressures, central venous pressure, pulmonary artery wedge pressure, and left ventricular diastolic pressure, the changes from the control value were calculated in mm Hg. Descriptive statistics were calculated for each variable at the intervals specified and will be presented as the mean and standard error of the mean.

To investigate the role of right ventricular failure in the declining cardiac output, we determined V_{max} (12) for the right ventricle during endotoxin shock, and compared this to V_{max} of the left ventricle. We believe that V_{max} is an index of contractility that is well-suited for following ventricular performance in endotoxin shock, since it is relatively insensitive to changes in afterload (13). The method does not require the determination of ventricular function curves, which requires interventions that may, *per se*, alter the target variable. For V_{max} determinations both right and left ventricle pressures were obtained in an additional 15 dogs. V_{max} was calculated from the isovolumic period of the respective ventricular pressure records, using a PDP 11/45. For the right ventricle, we used the same constants introduced for the left ventricle, 40 and 80 for K and C, respectively, in the formula V_{ce} (for the contractile element velocity of shortening) = $(dp/dt)/(KP + C)$. Normalization was performed for the values of V_{max} for each animal during the course of endotoxin shock before calculating the statistics, taking the control value as 100%.

To test the assumption that cardiac output in the dog could be restored by volume replacement, six animals were subjected to rapid infusions of low molecular weight Dextran, 25 ml/kg. The infusions were begun at five minutes after administration of endotoxin, and were completed prior to the fifteen-minute determination of cardiac output. (This would increase circulating volume during the interval of maximal hepatosplanchnic trapping) (2, 3). In addition, arterial PO_2 (PaO_2) was determined at the same intervals, using an Instrumentation Laboratories device. Comparison of mean values of these groups was done by unpaired *t* test.

Results. The hemodynamic course of early endotoxin shock is presented in Fig. 1. Cardiac output and mean pressures in the aorta, pulmonary artery, pulmonary artery wedge position, left ventricle, and central veins from twelve dogs are graphed for 4 hr. After endotoxin, there was a precipitous drop in cardiac output and aortic pressure, and a modest improvement by one hour. Thereafter, there

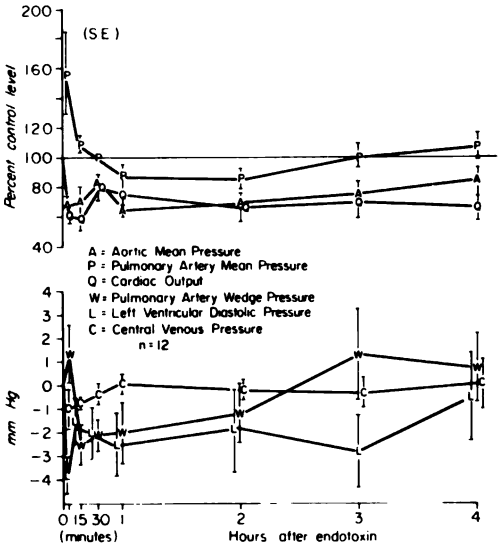

FIG. 1. Hemodynamic course in the first 4 hr of endotoxin shock in 12 dogs. The mean and SE of the mean are presented. Cardiac output (Q), aortic pressure (A), and pulmonary artery pressure (P) are presented as percentages of the control value. Pressures for pulmonary artery wedge (W), central venous pressure (C), and left ventricular diastolic pressure (L), are in mm Hg change from the control value for each animal.

was a gradual decline in output, but a gradual recovery of aortic pressure. In contrast to aortic pressure, the pulmonary artery pressure after endotoxin actually rose to over 150% of the control level; the pressure declined by one hour, and the two pressures paralleled each other after that with the normalized pulmonary pressures approximately 20% higher than the aortic. The pulmonary artery wedge pressure also rose in early endotoxin shock and fell by 15 min. It then commenced a very gradual recovery toward and above the control level by 4 hr. The left ventricular diastolic pressure had a prompt drop to a −3 mm Hg, recovered by 15 min to a −2 mm Hg, and remained at about that level until the 4-h determination. The central venous pressure fell less than 2 mm initially and recovered to almost control level for the entire experimental period.

The course of vascular resistance during endotoxin shock in the first 4 hr is presented in Fig. 2. The systemic vascular resistance is proportionately much lower than the pulmonary resistance, since the mean does not exceed 125% of control during the first 2 hr, whereas the peak pulmonary resistance is 450% greater than control.

Figure 3 presents the mean of V_{max} for the two ventricles and the cardiac output, normalized to the control value for each animal before averaging. The V_{max} for the left ven-

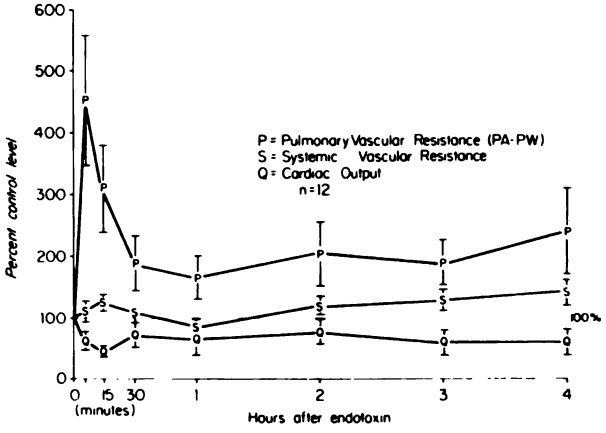

FIG. 2. Vascular resistances and cardiac output for the first 4 hr of endotoxin shock in 12 animals, presented as percentages of the control value.

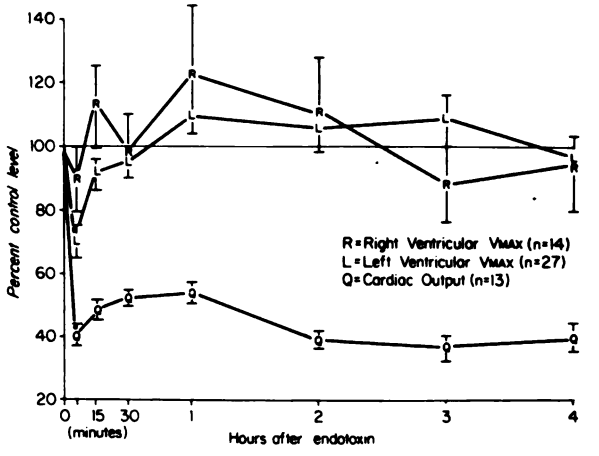

FIG. 3 Graphic comparison of an index of contractility, V_{max}, for the right (R) and left (L) ventricles, normalized with reference to the control value (100%) during endotoxin shock. For contrast, the cardiac output (Q) is presented. See text for significant differences.

tricle is initially more affected, but both ventricles recover to higher than control value by 1 hr. (The only statistically significant difference bewteen R and L V_{max} is at 15 min; $P < 0.01$). The left ventricular V_{max} remains greater than control for the first 3 hr, and falls slightly at the fourth hour; the index for the right ventricle has a greater variance for any single period, and the mean varies more from time to time, but there is no evidence in this data for failure of either the right or left ventricle to account for the very poor cardiac output during the first 4 hr.

The administration of 25 ml/kg of low molecular weight Dextran restored cardiac output to preendotoxin levels, but aortic mean pressure was restored only to 90% of control. The difference between output and pressure was also seen in the subsequent course. There was a significant difference ($P < 0.01$) in cardiac output between the fluid-treated group and the control group for the entire 4 hr, but the difference for the aortic mean pressures was significant ($P < 0.05$) at 30 min, one and 2 hr after endotoxin, but not by 3 or 4 hr. The arterial PO_2 for the untreated group fell briefly, but recovered to control level by 15 min, and remained above control thereafter. The difference in PO_2 between the two groups became significant by 2 hr, and was highly significant by 4 hr ($P < 0.01$), with the fluid-treated group showing deterioration. In addition, survival time was actually less in the animals treated with Dextran, although the differences were not significant. The dogs receiving dextran survived 8.5 hr, compared to 10.5 hr for the untreated.

Discussion. The course of the dog's pulmonary vascular resistance (PVR) after endotoxin is quite similar to the other species that have been reported, including the cat (4), sheep (6), calf (14), and primate (15). The marked changes in PVR have been neglected in the dog in favor of hepatosplanchnic trapping of blood (1). The duration of hepatosplanchnic trapping of blood, however, is less than 30 min (2), and relief by portocaval shunting fails to improve the hemodynamic course or survival (3). It is also of interest that endotoxin shock has a lethal course similar to the dog in animals that have no throttlelike mechanism in their hepatic veins (6, 15, 16). Detailed time course of pulmonary vascular changes in these other species have not been supplied in previous reports, but most publications suggest that the increases last only 30 min or less. We found that the marked elevation of resistance in the canine pulmonary vasculature was brief, and the resistance returned to moderate levels by 30 min after endotoxin (Fig. 2). This time course is strikingly similar to that of the portal vein pressure (3). These two vascular beds, pulmonary and splanchnic, also accumulate most of the endotoxin, judging from radioactive tagging, with the largest concentration in the lung, liver, and spleen (17). In short, the dog's pulmonary vasculature appears to be no less a target organ for endotoxin shock than in other species, and the brief hepatosplanchnic pooling is merely another vascular manifestation of this toxin, unique to the carnivores, but not uniquely lethal.

Halmagyi's inference of cor pulmonale in the sheep was based on an increase in right ventricular afterload that was relatively modest, a doubling of the mean pressure in the pulmonary artery (6). Although Halmagyi did not postulate a primary myocardial depression, this has been suggested recently for the canine left ventricle (10, 11) which has a substantially reduced afterload or aortic pressure. If the right ventricle were equally depressed, the effect should be more important, and more easily demonstrated, since the right ventricular afterload is roughly doubled. However, we do not find a significant reduction of myocardial contractility, using the index V_{max}, for either left or right ventricle during the first 4 hr, at which time the cardiac output was reduced drastically. Since our studies did not extend beyond 4 hr, we cannot speak to the late depression of myocardial contractility, described by Lefer's and Hinshaw's groups, after 4–8 hr. It is also possible that compensatory mechanisms in our intact animal preparation masked a direct myocardial depression. Two such compensatory mechanisms are catecholamine release and the positive inotropic effect of tachycardia, both of which are present in endotoxin shock due to baroreceptor activity. Regardless of how the contractility is maintained, the early vascular collapse cannot reasonably be attributed to cor pulmonale.

Although the dog's response to fluid ther-

apy has been used as evidence that vascular collapse in the dog is due to intravascular pooling (7), the transient nature of the improvement suggests that extravasation of the circulating volume is more important. Vascular leakage due to endothelial injury (18), enhanced by venoconstriction (2), continues throughout endotoxin shock, in the lung (19) as well as in the systemic circulation (20). This leakage accounts for the declining course of the arterial PO_2 after Dextran, compared to the PaO_2 in the control animals, and causes a therapeutic dilemma in the treatment of endotoxin shock. The administration of plasma or Dextran, not to mention saline or lactated Ringer's solution, restores flow and improves pressure, but the intravascular volume continues to be lost at the expense of the arterial PO_2.

Summary. The pulmonary vascular resistance in herbivores has been reported to increase threefold in endotoxin shock; poor cardiac output has been attributed to cor pulmonale. The poor cardiac output in dogs has been attributed to splanchnic pooling. However, we previously reported that splanchnic pooling was brief, and had resolved, while cardiac output remained low. In the present study of 27 anesthetized dogs we found a marked reduction in cardiac output and aortic pressure following endotoxin, and an increase in systemic resistance to only 125%. Pulmonary arterial resistance rose to a peak of 450% of normal at 5 min, but by 30 min had fallen to 200% of control. The relative contractility of the two ventricles, based on V_{max} was equal to or greater than control for most of the 4 hr. Although the afterload for the right ventricle is actually increased, in contrast to a reduction for the left ventricle, contractility for both ventricles was maintained. Treatment with Dextran transiently restored cardiac output, but caused a significant gradual reduction in arterial PO_2 as a result of extravasation.

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ɔmethacin: Relationship between Ulcerogenic and Anti-Inflammatory Properties (40108)

ffects of an Intestinal Lesion-Preventing Fat-Free Diet on Anti-Edema and Anti-Granuloma Properties of Indomethacin in the Rat

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activity and side effects of nonsteroidal flammatory agents (NSAI) are attrib- mechanism(s) involving common fac- , 2). Therefore, it seemed worthwhile ermine whether or not a fat-free diet , shown to reduce indomethacin-in- intestinal lesions (3) and toxicity (4), affect its anti-inflammatory activity. reliminary data indicated that FFD ıot reduce, but rather increases, this /. However, our initial experimental did not include the prostaglandin of inflammation, shown to be reduced fed an essential fatty acid-deficient).

iew of the above, we thought it worth- to determine the possible effects of ın the essential fatty acid pattern and nd our observation period to include ffects on the prostaglandin phase of mation.

erials and methods. Male albino rats, strain, weighing 110–130 g, were di- into groups of eight or more animals ıd fed either a regular or a fat-free diet 10-day period before commencing the nents, unless otherwise specified. In- ıacin was administered by gavage sus- l in an aqueous vehicle (NaCl, 0.9%; 80, 0.4%; CMC, 0.5%; and benzyl l, 0.9%).

regular diet (RD) consisted of 33.3% corn, 12.7% soybean flour, 12.7% meat 14.8% wheat bran, 4.5% barley flour, atmeal, 7.4% nonfat milk solids, 2.8% flour, 3.7% yeast, and 3.6% vitamins inerals. The fat-free diet (FFD) (6) ed of 30% vitamin-free casein, 64% 2% cellulose, and 4% vitamins and ls.

Effect of diet on growth curve. Two of 20 animals each were fed either a regular (RD) or a fat-free (FFD) diet over a 31-day period. The growth curve was recorded at several time intervals up to the end of the observation period.

(B) Carrageenin-induced paw edema. Immediately before the administration of indomethacin or the aqueous vehicle, each animal received 5 ml of water by gavage. One hour later, 0.1 ml of a 0.5% suspension of carrageenin (Rex 7205, Marine Colloids Inc., Springfield, N.J.) was injected through a 20-gauge needle into the plantar aponeurosis of the right hind paw (7). Foot volume was measured immediately following carrageenin administration and again 3 hr later by means of a mercury plethysmometer. This was done by immersing the paw of the unanesthetized animal up to the point where mercury was at the level of the lateral malleolus. The increase in paw volume was calculated from the difference in the values at 0 and 3 hr after the injection of the phlogistic agent.

(C) Effects on the essential fatty acid (EFA) pattern and the prostaglandin phase of inflammation. Animals were handled as described in B, with the exceptions that: (i) Paw edema was measured at 3 and 6 hr [prostaglandin phase (5)] after carrageenin administration; and (ii) groups of five animals each were used to determine plasma free fatty acids (FFA) and indomethacin, as well as the arachidonic (20:4) to eicosatrienoic (20:3) acid ratio in erythrocytes as a measure of the EFA pattern (8). Blood samples were obtained under ether anesthesia by heart puncture using a heparinized syringe. After centrifugation (30 min, 950*g*), plasma FFA were determined according to Ducombe (9), except that chloroform–heptane–methanol (49:49:2) was used as the extraction mixture. Plasma indomethacin was determined according to Kwan *et al.* (10). Arachidonic and eicosatrienoic acids

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were determined in erythrocytes' total fat through extraction (11) and alkaline isomerization (12).

(D) Cotton pellet-induced granuloma. Under light ether anesthesia, one cotton pellet was introduced subcutaneously on each side of the back through a midline incision. The pellets were cut from dental cotton rolls (No. 1, Johnson & Johnson) and paired to a combined dry weight of 100 ± 1 mg. Prior to implantation, the pellets were dipped for 1 min in a 1% suspension of carrageenin, placed on filter paper, and allowed to dry overnight under a lamp. Thereafter the pellets were autoclaved at 121°C for 1 hr (13). At the time of implantation, the pellets were individually soaked in a 1% solution of Pfizer Combiotic. Treatment started at the same time as implantation and lasted for 8 consecutive days. On the morning of the eighth day, immediately before the last treatment, each animal received 5 ml of water by gavage. One hour later, 0.1 ml of a 0.5% carrageenin suspension was injected through a 20-gauge needle into the plantar aponeurosis of the right hind paw. Thereafter the procedure is similar to that described in B. Immediately after measuring the paw volume, the animals were killed by CO_2 asphyxiation and the pellets with the surrounding granuloma were excised and dried overnight at 60°C. The amount in excess of 100 mg represents the weight of the granuloma, which was expressed either in absolute values or in mg/100 g body weight (14).

(E) Three-day carrageenin paw edema (15). This modification of the method outlined in B was carried out as follows.

(i) Animals were divided into two groups of 20–22 rats each and were fed either a regular (RD) or a fat-free (FFD) diet. Fourteen to fifteen animals of each group received oral indomethacin (18 mg/kg) in three divided doses of 6 mg each, at 42, 30, and 1 hr before carrageenin. Seventeen hours after measuring the degree of swelling, the animals were autopsied and the intestine was examined for the presence of ulceration by an observer who was unaware of the treatment.

(ii) Animals were divided into six groups of 8 rats each and fed a regular diet (RD). Indomethacin was given orally in single doses of 6 mg/kg each at different time intervals before carrageenin, as specified in Table VI. This technique of dosing, which differs from that outlined in (i), was aimed at elucidating to what extent the anti-edema properties of indomethacin were due to a specific (anti-inflammatory) or to a nonspecific (ulcerogenic) effect. Seventeen hours after measuring the degree of swelling, the animals were autopsied and the intestine was carefully and thoroughly examined for the presence of inflammation site(s) and/or ulceration by an observer who was unaware of the treatment. For a better evaluation of the specific to nonspecific anti-edema ratio, the degree of intestinal pathology was graded according to the scale shown in Table VI.

(F) Statistical procedures. The data relative to carrageenin-induced paw edema were calculated as adjusted values derived from an analysis of covariance, where the variates x and y were the paw volume before carrageenin and the increase in paw volume at 3 and 6 hr after the injection of the phlogistic agent, respectively. Contrasts among adjusted means were calculated according to Snedecor and Cochran (16).

The data relative to granuloma weight, plasma concentration of FFA, or indomethacin and arachidonic acid content in erythrocytes were calculated by means of an analysis of variance. When the test indicated significant F values, an inspection of all differences between pairs of means was made according to the LSD method (17).

Percentage inhibition values as well as average body weight data were subjected to Student's t test unless otherwise specified in the tables. The data relative to incidence and degree of intestinal ulcers were analyzed by means of the χ^2 test (corrected for continuity) and the Wilcoxon test (18), respectively, unless otherwise specified in the tables. The correlation between indomethacin-induced body weight changes, anti-edema activity, and degree of intestinal pathology was calculated according to the Spearman rank coefficient (19).

Results. (A) Effect of diet on the growth curve. The data in Table I indicate that, from Day 10 on, FFD significantly reduced the growth rate in the male rat.

(B) Carrageenin-induced paw edema. Statistical analysis of the data in Tables II and III indicates that: (i) Indomethacin significantly reduces carrageenin-induced paw

TABLE I. EFFECT OF DIET ON THE GROWTH CURVE OF MALE RATS.

Experimental design	No. of animals	Body weight in grams (mean ± SE) on Days: 0	10	17	24	31
Regular diet (RD)	20	135.3 ± 1.8	193.0 ± 4.3	227.2 ± 6.2	259.1 ± 6.4	284.5 ± 5.8
Fat-free diet (FFD)	20	135.3 ± 1.8	179.0 ± 6.1	198.3 ± 6.5*	213.1 ± 7.8*	232.9 ± 10.5*

* $P < 0.01$.

TABLE II. EFFECT OF DIET AND ORAL INDOMETHACIN (5 mg/kg) ON CARRAGEENIN-INDUCED PAW EDEMA.

Experimental design	No. of animals	Increase in paw volume after carrageenin: Adjusted mean	Percentage inhibition
Regular diet (RD)			
Indomethacin	8	13.2	30
Controls	8	19.0	
Fat-free diet (FFD)			
Indomethacin	8	8.0	52.1*
Controls	8	17.1	
Least significant difference			
$P < 0.05$		5.9	
$P < 0.01$		8.0	

* $P < 0.05$ compared to RD group.

edema in both RD and FFD rats; (ii) the degree of inhibition is significantly greater in FFD as compared to RD animals.

(C) Effects on the essential fatty acid (EFA) pattern and the prostaglandin phase of inflammation. Statistical analysis of the data in Table III indicates that: (i) Six hours after carrageenin, the paw edema volume is identical in RD and FFD control rats. (ii) Six hours after carrageenin, the paw edema volume of indomethacin-treated RD and FFD rats is significantly lower than that of control animals. (iii) The arachidonic acid content in erythrocytes is independent of the nature of the diet, carrageenin, or indomethacin treatment. There is no detectable amount of eicosatrienoic acid in either RD or FFD rats. (iv) Independently of diet and indomethacin, the plasma FFA concentration is significantly increased at 3, but not at 6, hr after carrageenin. This increase is significantly greater in FFD as compared to RD rats. (v) There is no detectable difference in plasma concentration of indomethacin between RD and FFD rats at either 3 or 6 hr after carrageenin.

(D) Cotton pellet-induced granuloma. Statistical analysis of the data in Table IV indicates that indomethacin significantly reduces granuloma formation and carrageenin-induced paw edema in both RD and FFD rats, the degree of inhibition being significantly greater in FFD as compared to RD animals.

(E) Three-day carrageenin paw edema. (i) Statistical analysis of the data in Table V indicates that: (a) Indomethacin significantly reduces carrageenin-induced paw edema in both RD and FFD animals, the degree of inhibition being significantly greater in RD as compared in FFD rats. (b) Decrease in body weight (limited to RD rats) is associated with greater anti-inflanmatory activity, as indicated by the close relationship ($P < 0.001$) between body weight changes determined immediately before the last dose of indomethacin and anti-edema activity. (c) Both the incidence and degree of indomethacin-induced intestinal lesions are significantly greater in RD as compared to FFD rats.

(ii) Statistical analysis of the data in Table VI indicates that: (a) Indomethacin administered 42 hr before carrageenin does not influence paw edema formation. (b) Indomethacin administered either at 42 and 30, at 30 and 1, or at 1 hr before carrageenin produces a significant but similar antiedema effect. Such an effect is significantly lower than that observed following indomethacin administered at 42, 30, and 1 hr before carrageenin. (c) There is a close relationship (Fig. 1) between anti-edema activity, degree of intestinal pathology, and body weight changes. The latter were determined immediately before the last dose of indomethacin.

Discussion. Reduction and/or prevention of indomethacin-induced intestinal lesions has been achieved by a variety of experimental conditions, such as fasting (3, 21), fat-free diet (3, 4), various catatoxic steroids (22, 23), prostaglandins (24), cholestyramine (21), low-residue diet, (25) and antibiotics (26).

With the exception of spironolactone, which has been shown to reduce both intes-

TABLE III. EFFECT OF DIET AND ORAL INDOMETHACIN (5 mg/kg) ON CARRAGEENIN INDUCED PAW EDEMA, PLASMA CONCENTRATION OF FREE FATTY ACIDS (μmole/ml), AND INDOMETHACIN (μg/ml) AND ARACHIDONIC ACID CONTENT IN ERYTHROCYTES (PERCENTAGE OF TOTAL LIPIDS).

Experimental design	Time after carrageenin (hr)	No. of animals	Paw volume increase after carrageenin: Adjusted mean	Paw volume increase after carrageenin: Percentage inhibition	No. of animals	Plasma free fatty acids[b]	Erythrocyte arachidonic acid[b]	Plasma indomethacin[b]
Regular diet (RD)								
Indomethacin	3	14	8.8	45.6	5	2.64 ± 0.57	7.7 ± 1.9	14.2 ± 1.1
	6	9	10.6	36.0	5	1.08 ± 0.28	9.2 ± 2.0	8.3 ± 1.4
	0	—	—	—	5	0.58 ± 0.15	8.0 ± 1.4	—
Controls	3	15	16.1	—	5	2.85 ± 0.42	11.7 ± 3.6	—
	6	10	16.6	—	5	1.09 ± 0.57	9.0 ± 1.8	—
Fat-free diet (FFD)								
Indomethacin	3	15	7.8	60.1*	5	4.1 ± 0.57	12.3 ± 2.3	14.3 ± 1.6
	6	10	12.2	28.0	5	1.01 ± 0.25	6.6 ± 2.0	10.7 ± 1.5
	0	—	—	—	5	0.76 ± 0.22	9.9 ± 0.2	—
Controls	3	15	19.7	—	5	4.12 ± 0.46	10.4 ± 2.4	—
	6	10	16.5	—	5	1.1 ± 0.28	5.2 ± 1.1	—
Least significant difference[a]								
$P < 0.05$			2.5 (4.0)			1.17		2.6
$P < 0.01$			3.3 (5.3)			1.57		3.5

[a] Those relative to paw volume increase at 6 hr are in parentheses.
[b] Mean ± SE.
* $P < 0.05$ as compared to paw volume increase of RD group at 3 hr.

TABLE IV. EFFECTS OF DIET AND ORAL INDOMETHACIN (2.5 mg/kg/day) ON GRANULOMA FORMATION AND CARRAGEENIN-INDUCED PAW EDEMA.

Experimental design	No. of animals	Granuloma weight (mean ± SE): Absolute wt (mg)	Granuloma weight (mean ± SE): Milligrams per 100 g body wt	Percentage inhibition: Absolute wt (mg)	Percentage inhibition: Milligrams per 100 g body wt	Increase in paw volume after carrageenin: Adjusted mean	Increase in paw volume after carrageenin: Percentage inhibition
Regular diet (RD)							
Indomethacin	8	107.1 ± 7.9	56.2 ± 3.6	30.5	26.0	8.7	42.4
Controls	8	154.1 ± 19.8	75.9 ± 9.0			15.1	
Fat-free diet (FFD)							
Indomethacin	8	93.4 ± 6.2	53.9 ± 4.6	43.2	41.2*	4.1	72.4*
Controls	8	164.4 ± 9.8	91.6 ± 7.0			14.9	
Least significant difference							
$P < 0.05$		36.9	18.5			2.9	
$P < 0.01$		49.8	25.0			3.9	

* $P < 0.05$ compared to RD group.

tinal lesions and anti-inflammatory activity (27), no effort has been made to determine whether or not a reduction in intestinal lesions is accompanied by a concomitant modification in anti-inflammatory activity.

Our data indicate that FFD prevented and/or reduced intestinal ulcers without reducing, but rather enhancing, the anti-inflammatory properties of indomethacin. This enhanced activity, observed in models of both chronic and acute inflammation, but particularly evident in the latter, could be attributed to differences in body distribution of indomethacin between RD and FFD rats, due to a dissimilarity in protein binding. This hypothesis is consistent with data indicating that:

(i) There is a correlation (28, 29) between protein-bound indomethacin (30, 31) and anti-inflammatory activity.

(ii) Any increase in plasma FFA concentration ranging from 2 to 4 m*M* (32), like that observed in FFD animals, has been associated with the displacement of protein-bound NSAI (32–34).

(iii) A direct involvement of catecholamines (35) and/or corticosterone in enhancing the anti-inflammatory activity of indomethacin in FFD rats seems unlikely, since increase in paw volume of the untreated animals was independent of the nature of the diet.

(iv) An indirect involvement of corticosterone in enhancing the anti-inflammatory activity of indomethacin in FFD animals through an indomethacin-induced modification in the free to protein-bound corticosterone ratio (36) seems likewise unlikely in view of Winter's findings (37).

The fact that we were unable to detect any variation in total plasma concentration of indomethacin between RD and FFD rats is not at variance with this hypothesis, since total drug concentration in plasma appears to

TABLE V. EFFECT OF ORAL INDOMETHACIN (18 mg/kg IN THREE DIVIDED DOSES) ON CARRAGEENIN-INDUCED PAW EDEMA AND INCIDENCE AND DEGREE OF INTESTINAL ULCERS.

Experimental design	No. of animals	Mean body weight (g)[a]	Increase in paw volume after carrageenin		Intestinal ulcers	
			Adjusted mean	Percentage inhibition	Percentage incidence	Degree[b] (mean score)
Regular diet (RD)						
Indomethacin	14	−11.1 ± 1.8	2.2	86.2	100	1.9
Controls	8	—	16.4		—	
Fat-free diet (FFD)						
Indomethacin	12	+3.5 ± 1.8	9.5	53.7*	25*	0.4*
Controls	8	—	21.0		—	
Least significant difference						
$P < 0.05$			3.3			
$P < 0.01$			4.5			

[a] From Day 1 to Day 3 (before the third administration of indomethacin); mean ± SE.

[b] Degree of intestinal ulceration was graded according to an arbitrary scale: 0 = normal, 1 = primary ulcers, 2 = advanced ulcerative processes, 3 = perforating ulcer and intestinal adhesions.

* $P < 0.01$ compared to RD group.

TABLE VI. EFFECT OF DOSING SCHEDULE OF ORAL INDOMETHACIN ON CARRAGEENIN-INDUCED PAW EDEMA AND INCIDENCE AND DEGREE OF INTESTINAL LESIONS.

Indomethacin (mg/kg) at the following hours before carrageenin			No. of animals	Δ body weight (g)[a]	Increase in paw volume after carrageenin		Intestinal pathology		
					Adjusted mean	Percentage inhibition	Percentage incidence of ulcers	Degree[b]	
42	30	1						Mean score	Mean rank
—	—	—	8	+18.3 ± 1.2	20.7				
6	—	—	8	+17.3 ± 1.0	19.1	9.3	0	0.25	12.0
6	6	—	8	−2.9 ± 2.6	9.4	54.6	62.5	3.25	25.6
6	6	6	8	−0.4 ± 2.2	2.8	86.6	100	5.13	33.8
—	6	6	8	+13.8 ± 3.0	10.4	49.7	50	2.0	19.1
—	—	6	8	+16.7 ± 1.5	11.6	44.0	12.5	0.5	11.9
Least significant difference									
$P < 0.05$				5.8	4.5		40		16
$P < 0.01$				7.8	6.0		55		19

[a] From Day 1 to Day 3 (before the third administration of indomethacin); mean ± SE. Data were analyzed by means of a one-way analysis of variance. Multiple comparisons among groups were made by means of the LSD method (17).

[b] Degree of intestinal pathology was graded according to an arbitrary scale: 0 = normal, 1 = irritation, 2 = marked irritation, 3 = one or two small ulcers, 4 = several primary ulcers, 5 = many advanced ulcers, 6 = many ulcers, with adhesions, 7 = death. Scores were reduced to ranks and analyzed by means of a Kruskal–Wallis one-way analysis of variance (20). Multiple comparisons among groups were made by means of the critical range method (20).

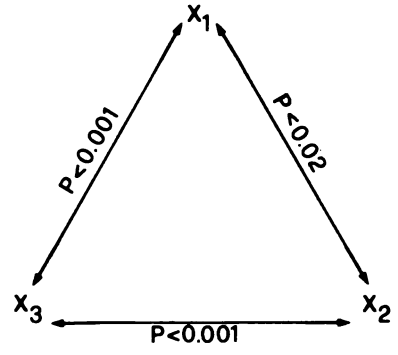

FIG. 1. Spearman rank coefficients of the values shown in Table VI relative to body weight changes (X_1, increase in paw volume (X_2), and degree of intestinal pathology (X_3).

be a relatively poor index of changes in drug distribution (38). For example, if indomethacin is 99% protein-bound (37) and is 5% displaced, this will result in a negligible and undetectable variation in total plasma concentration, but in a significant sixfold increase in the amount of drug free to enter the sites of action.

Further indirect evidence for this hypothesis is provided by the fact that FFD failed to enhance the anti-inflammatory activity of indomethacin during the prostaglandin-mediated phase of inflammation. Such a failure cannot be attributed to a modification in prostaglandin availability as indicated by the similarity in EFA pattern and paw edema response between untreated RD and FFD rats during this phase, but rather to restoration of plasma FFA to normal levels.

In view of the data obtained thus far, the finding that, in the 3-day carrageenin paw edema, indomethacin was significantly more effective in RD as compared to FFD animals was rather unexpected. However, the almost-complete inhibition of paw edema formation observed in RD rats following the three doses of indomethacin has to be attributed to the combined anti-inflammatory effect of specific and nonspecific factor(s). That is, the effect (specific) of the last dose of indomethacin superimposes on the effect (nonspecific) produced by the preexisting inflammatory processes at intestinal levels apparent in RD, but not FFD, animals.

The nonspecific role played by the preexisting inflammatory processes produced by the administration of the first two doses of indomethacin is substantiated by the f ing:

(i) The presence of indomethacin body, following single or repeated ad trations (42 and 30 hr before the injec the phlogistic agent), at the time of geenin injection is against kinetic con tions (39, 40).

(ii) Paw edema formation is signif reduced in the presence of preexisting i matory processes, as indicated by ou data and previous findings (15, 41). Th specific anti-edema effect does not seen due (42) to the production, at pree inflammatory sites, of a factor(s) capa inhibiting inflammatory processes at sites upon release into the blood stre appears more likely to be attributable, in part, to a reduction in serum kini complement (15) rather than to a defi in prostaglandin availability (24), sin effect is evident in the non-prostaglandi diated phase of inflammation (5).

In view of the above, it appears legi to conclude that FFD prevents and or r intestinal lesions without reducing, but enhancing, the specific anti-inflammat tivity of indomethacin. This is at va with the hypothesis that anti-inflamn activity and side effects have a comm nominator (1, 2).

This observation is of clinical rele and it is tempting to speculate that fee low-fat diet might reduce NSAI-induce trointestinal disturbances without re therapeutic efficacy.

Summary. The anti-edema propert ulcerogenic doses of indomethacin shown to be due to the concomitant infl of specific (anti-inflammatory) and n cific (presence of intestinal lesions) e The fat-free diet (FFD) prevented a reduced both the incidence and deg indomethacin-induced intestinal lesions out reducing, but rather enhancing, a flammatory activity through a specific

These findings are at variance wi hypothesis that anti-inflammatory a and side effects of indomethacin have mon denominator. The greater anti-i matory activity displayed by indometh FFD as compared to regular diet (R rats might be attributed to differences i distribution of the drug. It is tempt

speculate that feeding a fat free or a low fat diet might reduce NSAI-induced gastrointestinal disturbances without reducing therapeutic efficacy.

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N-Acetylneuraminic Acid (NANA) in Measles Virus (40109)

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The envelope glycoproteins and glycolipids of paramyxoviruses appear to be derived from the host cell plasma membrane during viral release (1, 2). Measles envelope glycoprotein spikes effect attachment of the virion to cell surfaces, thereby initiating either hemagglutination (HA) or infection (3, 4). Most viruses which mature at the cell surface incorporate NANA from the plasma membrane (5–9). Myxoviruses and paramyxoviruses do not do so, because they contain virus-coded neuraminidase (10–12). NANA residues are detectable on purified vesicular stomatitis virus (VSV) (5, 8, 12, 13), avian tumor (7), rabies (9), Sindbis (14, 15) and Semliki Forest viruses (16), as well as on temperature-sensitive mutants of influenza virus type A which lack neuraminidase (17). In this report, we present evidence to show that measles virions likewise possess NANA residues which are involved in the adsorption of virus to the surface of cells.

Materials and methods. VERO cells, obtained from the American Type Culture Collection, were grown in Eagle's minimal essential medium (MEM) in Earle's balanced salt solution (BSS) with 10% heat-inactivated fetal calf serum (FCS) and were maintained in the same medium with 2% FCS. The Edmonston strain of measles virus, grown in VERO cells, was partially purified from infected culture fluids by banding in a 20–40% sucrose gradient in a swinging bucket rotor (SW 27) at 80,000*g* for 3 hr. Parainfluenza virus type 2 (18) and VSV were propagated in VERO cells. Influenza virus was grown in 11-day old embryonated eggs, and mumps virus in primary cultures of chicken embryo fibroblasts.

Plaque titrations were carried out on confluent monolayers of VERO cells in 35 mm plastic dishes (Falcon), using a single overlay of 1% agar in maintenance medium. Plates were stained with neutral red 6 days after infection and the plaques counted tw later. Rhesus erythrocytes,[2] freshly into dextrose-citrate, washed three tim resuspended to 0.5% in phosphate-b (pH 7.2) saline (PBS), were used for hemagglutination and inhibition assa measles virus. To measure inhibition o enza virus hemagglutination (19), seri bling dilutions of measles virus in PB mixed with equal volumes of influe virus containing 4 HAU, followed by volumes of 0.5% chicken erythrocyte HI titer was taken as the highest dilut measles virus which inhibited 4 HAU fluenza virus.

Protein was determined by the met Lowry (20) with bovine serum albu standard. *N*-acetylneuraminic acid (N was determined by the method of V (21) using crystalline NANA as standa

Results. As shown in Table I, measle pelleted from infected culture fluids slightly higher NANA: protein ratio washed and gradient-purified virus. T tios in all viral preparations, however higher than those found in normal V cells. Measles virus (6×10^6 PFU) and (5×10^7 PFU) were each treated for 9 at 37° with *Vibrio cholerae* neurami (Behring Diagnostics) at a concentrat 20 U/ml in Tris–HCl buffer, pH 7.0 co ing 0.5 μmoles $CaCl_2$. After incubatio virus–enzyme mixtures were chilled, th was pelleted by centrifugation for 90 125,000*g*, washed once and then resusp in PBS to the original starting volume. virus-free supernatants were saved fo ysis. Results are shown in Table II. natants from neuraminidase-treated n virus were found to contain approxi 80% of the hydrolyzable NANA fou pelleted virions not previously expo enzyme. No chromogen was found in drolyzed supernatants of untreated vir

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[2] Supplied by the Delta Regional Primate R Center, Covington, Louisiana.

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sidual chromogen could be detected in hydrolyzates of neuraminidase-treated es virus. Supernatants from neuramin- treated virions contained no more pro- neasurable by the Lowry method than natants from untreated virus (Table II), ıting that the neuraminidase prepara- used had no contaminating protease ty. Acid hydrolyzates of untreated VSV ined substantial amounts of chromogen. ANA was found in hydrolyzed influ- A virus. Measles virus and VSV each ted influenza HA significantly. Neura- ase treatment abolished this inhibition ll as the infectivity of both viruses. les virus treated with either neuramini- r trypsin no longer agglutinated rhesus ocytes and retained virtually no infec-

rder to determine the effect of enzymes riodate treatment on susceptibility to ion, monolayers of VERO cells in 35 lates were treated at 37° with *V. chol-* neuraminidase (Worthington) (20), trypsin (1 mg/ml), pronase (1 mg/ml) taperiodate (0.011 *M*) for different pe- of time up to 60 min. After treatment, in each case caused only minimal ling and no detachment of cells, the layers were washed three times with 5 S, infected with serial tenfold dilutions asles virus, overlaid with agar and in- ed. Staining and estimation of plaques then carried out as described.

shown in Fig. 1, treatment of cells with trypsin or pronase before infection re- by 60–70% the number of plaques should have appeared in comparison treated controls. After 10 min of perio- reatment, the plaque count was reduced out 90% as compared with untreated ls; and no plaques appeared in cells had been treated for 60 min. In con- pretreatment up to 30 min with neura- ase appeared to enhance slightly the tibility of cells to infection.

throcytes were treated with the same nts in order to determine the effect on ceptors. Cells were suspended in 0.011 dium metaperiodate ($NaIO_4$) and al- to stand at room temperature for 30 after which an equal volume of 40% se was added to stop the reaction. Eryth- s in 10% suspension were also treated with *V. cholerae* neuraminidase (Behring), 40 U/ml, for 1 hr at 37°, or with pancreatic trypsin (1 mg/ml, Difco 1:250) for 30 min at room temperature. Treated cells were washed three times with PBS and resuspended to 0.5% in PBS, and then tested for agglutinability by virus. The results, not otherwise given in detail, showed that desialylation of rhesus erythrocytes enhanced measles virus HA titers 8- to 16-fold. Influenza A virus, which agglutinates normal rhesus erythrocytes weakly, did not agglutinate neuraminidase-treated rhesus erythrocytes. Pretreatment of rhesus erythrocytes with either trypsin or periodate completely eliminated their agglutinability by measles virus. Human erythrocytes remained inagglutinable by measles virus after treatment with either neuraminidase or trypsin.

Discussion. We have shown that measles virus, which lacks neuraminidase activity and matures by budding from the cell surface (22, 23), contains a chromogen which has an adsorption maximum identical to that of NANA (Table I) and which is released from concentrated purified measles virions by the action of exogenous neuraminidase (Table II). Although most of this NANA undoubtedly stems from measles virus particles, the possibility cannot be excluded that some portion of the NANA detected in gradient purified virus may have been derived from frag-

TABLE I. NANA CONTENT OF MEASLES VIRUS

	Micrograms NANA[a]	Milligrams protein[b]	Ratio NANA/protein × 10^3
VERO cells[c]	40	18	2.2
Measles virus[d] pelleted from medium containing			
2% FCS	4.8	1.4	3.4
5% FCS	5.6	1.6	3.5
2% FCS and washed 2× in PBS	4.3	1.4	3.1
2% FCS, washed 2×, banded in sucrose gradient (20–40%), repelleted	4.0	1.3	3.1

[a] Determined by thiobarbituric acid procedure (28) after hydrolysis 30 min 80°C in 0.1 *N* H_2SO_4 referred to crystalline NANA.

[b] Lowry method referred to bovine serum albumin (20).

[c] 1×10^6 cells.

[d] 6×10^5 PFU used for each analysis.

TABLE II.

Virus	Concentration of NANA (μg/ml)	Concentration of Protein (mg/ml)	Influenza viral HI titer[a]	HA titer	Infectivity (PFU treated/PFU untreated)
Measles					
Neuraminidase treated[b]					
Pellet	0[c]	1.1	0	0	0.05
Supernatant	28	0.1			
Trypsin treated (Pellet)				0	0.003
Control					
Pellet	37[c]	1.2	16–32	32	
Supernatant	0	0.1			
VSV					
Neuraminidase treated	nd	nd	0	nd	0.002
Control	78[c]	1.6	128		

[a] Mumps virus gave no inhibition.

[b] PFU treated virus/PFU untreated virus, titered simultaneously. In four experiments with neuraminidase, mean titer of treated virus was $4.2 \times 10^4 \pm 2.2$, untreated virus $4.6 \times 10^6 \pm 2.1$, $P = <0.01$.

[c] After hydrolysis in 0.1 N H_2SO_4, 80°C, 30 min.

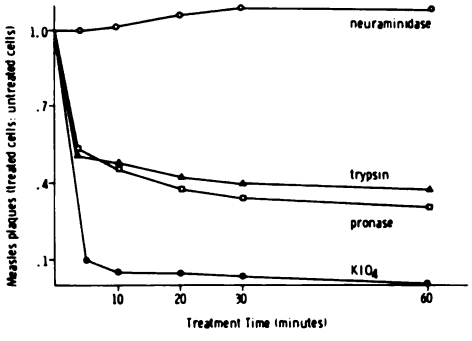

FIG. 1. Effect of pretreatment of VERO cells with neuraminidase, trypsin, pronase or periodate on susceptibility to infection with measles virus.

ments of infected host cell membrane containing predominantly virus-coded antigen as well as from defective, noninfectious particles (24) which might have cosedimented with complete virions. The latter two sources of NANA are still of viral origin. The amount of NANA absorbed to virions from calf serum in the medium was negligible.

Viruses which contain sialoglycoproteins have been reported to form aggregates (17) and to exhibit receptor activity for influenza virus, demonstrable by inhibition of hemagglutination (19). On this basis, our results provide further evidence for the presence of sialic acid on the measles virion, in contrast to mumps virus, which, because it bears intrinsic neuraminidase, lacks NANA and therefore fails to interact with influenza virus HA. Trypsin reduced both infectivity and HA to a greater extent than did neuraminidase, suggesting that the NANA required for these activities is attached to glycoprotein rather than to glycolipid.

Studies on VSV (25, 26) and those reported to date on measles virus (4) indicate that envelope glycoproteins mediate adsorption of viral particles to cells. NANA may play a central role in this process, which initiates infection in Vero cells and effects hemagglutination. Accordingly, infectivity was reduced and HA was eliminated by treatment with neuraminidase. That other as yet unidentified components of the viral envelope may be essential to attachment is suggested by the finding that trypsin treatment completely abolished infectivity, whereas some infectivity remained after neuraminidase treatment. This is consonant with the finding that trypsinization of desialylated VSV was reported to result in a 100-fold decrease in infectivity (26), suggesting that portions of the sialopeptide proximal to terminally linked NANA had some part in attachment of virions to cell receptors. The role of NANA in VSV infectivity is still not clear, however, since other workers have found that three different strains of VSV, after being stripped of NANA, remained fully infective for BHK and mouse cells, but lost HA activity (27). NANA appears not to be involved in the attachment of other enveloped RNA viruses to cells. Both Sindbis (25) and Semliki Forest viruses (16) were reported to retain biological function after treatment with neuraminidase. Sindbis virus grown in *Aedes albopictus* cells which lack NANA, was biologically equiva

) virus produced in either duck or ham-
:lls (14). It is apparent that no general-
ıs are possible regarding the role of
NANA in the attachment of virions to
usceptible to infection.

:ontrast to its essential role in the mea-
irion, the presence of NANA in the
)rotein receptor site appears not to be
ial for viral attachment. Accordingly,
ıent of VERO cells with neuraminidase
:d in moderate increase in their capacity
orb virus; and neuraminidase treatment
:sus erythrocytes markedly enhanced
:inability by virus. It is possible that
'al of NANA residues may unmask re-
's, and, by reducing the negative surface
:, promote attachment of measles viri-
Trypsin, pronase or periodate each
y reduced the susceptibility of Vero cells
ection, and abolished the reactivity of
; erythrocytes with measles HA. Oxi-
ı of terminal sugar residues, including
A, may account for the more pro-
:ed effects of periodate as compared
)roteolytic enzymes in reducing the ca-
' of VERO cells to absorb virus and in
ıating hemagglutination. These findings
ıte that the receptor groupings required
ral attachment, while not exclusively in
ıal linkage, are still distal to the site of
c cleavage.

tly, the residual bioactivity seen after
ıent of cells with proteolytic enzymes
l) may be a reflection of the experimen-
ıditions or it may indicate that the virus
: to utilize active sites on the cell surface
ı are not affected by the enzymes tested.

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Biological Evaluation of Crystalline Fraction I Protein from Tobacco[1] (40110)

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Leaf proteins are one of the most abundant protein sources in the world but at present very little leaf protein is used directly for human consumption. Proteins constitute but a small portion of the leaf; and for them to serve as a significant source of protein for man, they must be concentrated. The cost of such concentration has priced these proteins out of serious consideration as a source of human protein in the past. Technological improvements in the concentration of leaf proteins, however, have raised the possibility that leaf proteins may serve as an important source of protein for man in the future.

The distribution of total leaf protein is roughly 50% soluble and 50% insoluble in water. Of the former, approximately one-half consists of a single homogeneous protein whose molecular weight is approximately 500,000 daltons. This protein was called Fraction I protein at the time of its discovery in 1947 but has since been shown to be identical to the enzyme ribulose diphosphate carboxylase (1). This enzyme catalyzes the union of carbon dioxide with other carbon containing compounds during photosynthesis. It is present in all green leaves and is the most abundant, single protein in the world (1). Fraction I protein from tobacco is unique from that derived from other green plants in that only from plants of the genus Nicotiana (of which tobacco is a member) can it be obtained in pure, crystalline form (2). In the salt free, crystalline state, lyophilization produces a tasteless white powder. With the exception of a slightly lower level of methionine its content of essential amino acids equals or exceeds that of the FAO Provisional Pattern (Table I). In the present communication data are presented on the comparative nutritive value of casein and crystalline Fraction I protein from tobacco when fed as the sole source of dietary protein on the weight increment, PER (protein efficiency ratio), hematological findings, serum levels of protein, lipids and other serum constituents, and organ weights of immature male rats.

Materials and methods. Tobacco plants (Nicotiana tabacum) were grown in a green house and were harvested in 2–6 kg fresh-weight batches when they had attained a height of 18–21 inches. Immediately after harvesting, crystals of Fraction I protein were obtained by a slight modification of the method developed by Lowe (3).[2] The tobacco leaves were extracted with 0.1 *M* NaCl and beta mercaptoethanol, the filtered juice heated to 50° for 10 min, centrifuged to remove the insoluble green fraction, and the remaining brown solution passed through a G-25 Sephadex chromatographic column equilibrated with 0.025 *M* Tris–HCl and 0.0005 *M* EDTA, pH 7.6. The soluble leaf proteins eluted in the void volume which was collected. Fraction I protein crystals appeared in the eluate almost immediately and continued to appear during 3 days storage at 5°. The mother liquor was decanted from the layer of crystals which were resuspended and washed in a small volume of glass-distilled water. The suspensions containing 6–18 g (dry weight) of Fraction I protein crystals were examined by phase and dark field microscopy to insure the absence of bacteria. Enough 1 *M* NaCl was added dropwise to completely dissolve the crystals, the solution centrifuged to remove a slight turbidity and the clear, colorless solution placed in cello-

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[2] The authors are indebted to Dr. Richard Lowe of the University of Kentucky for having made his simplified method for isolating Fraction I protein crystals available for their use prior to publication.

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I. ESSENTIAL AMINO ACIDS IN CRYSTALLINE CTION I PROTEIN FROM TOBACCO LEAVES ED TO FAO PROVISIONAL PATTERN (GRAMS PER 100 GRAMS OF PROTEIN).

no acids	Provisional pattern[a]	Fraction I protein from tobacco[b]
ucine	4.2	4.2
ine	4.8	8.8
ie	4.2	5.8
ylalanine	2.8	4.4
sine	2.8	4.9
ionine	2.2	1.6
onine	2.8	5.2
tophan	1.4	1.5
ie	4.2	7.2

erence (11).
ulated from amino acid analyses contained in e (12).

tubing and dialyzed at 5° against 10 lass-distilled water which was changed s at 24-hr intervals to remove NaCl. crystallized protein was transferred to nd the protein lyophilized to produce e, nonhygroscopic powder. Approxi- 800 g of dry Fraction I protein crystals obacco were prepared for the present The latter was obtained from the proc- of 300 kg of fresh tobacco leaves.

nty male rats of the Sprague–Dawley ranging from 48–52 g in body weight ivided into two comparable groups of nals each. These were fed diets similar AOAC Protein Evaluation Diets (4) fered as to the source of protein. Rats ip I were fed ANRC Reference Casein[3] ose in group II Fraction I protein from o as the sole source of dietary protein was incorporated at a 10% level (based ontent × 6.25) in the test diets. Sucrose nployed as the source of dietary car- rate. Animals were placed in individual cages with raised screen bottoms and rovided the above diets and water *ad* . Animals were fed daily, and all food nsumed 24 hr after feeding was dis- . Food consumption was determined or each rat for 28 days. Animals were d once weekly. After the 28th day of g, rats were fasted overnight. In the ng they were anesthetized with sodium barbital, and 5 ml of blood was with- from each rat by heart puncture. Two ml were taken for hematological determinations; serum was separated from the remaining 3 ml and analyzed with a Technicon SMAC high speed computer controlled Biochemical Analyzer[4] for serum levels of protein, lipids and other serum constituents. The internal organs were examined grossly, and organ weights were determined. The data were treated statistically by Student's *t* test (5).

Results. Weight increment and PER. The average weight increment and PER of rats in the two dietary groups after 7, 14, 21 and 28 days of feeding are indicated in Table II. Both the weight increment and PER of rats fed the diet containing crystalline Fraction I protein from tobacco (group II) were significantly greater at each of the above time periods than that of rats fed the casein-containing diet (group I).

Hematological findings. The hematological findings of rats in the two dietary groups after 28 days of feeding are indicated in Table III. No significant differences in Hb, Hematocrit, RBC, WBC, MCHC, MCH, MCV and differential cell count were observed between the two groups.

Serum levels of proteins, lipids and other constituents. Serum levels of protein, lipids and other constituents for rats in the two dietary groups after 28 days of feeding are indicated in Table IV. Serum levels of protein, cholesterol, triglyceride, glucose, urea, creatinine, total bilirubin, inorganic phosphorus, sodium and chloride did not differ significantly between the two groups. Serum level of albumin, the A/G ratio, calcium and carbon dioxide were slightly but significantly greater in rats fed the diet containing crystalline Fraction I protein from tobacco than in rats fed the casein-containing diet. Serum globulin and potassium levels, however, were slightly but significantly greater in rats fed the casein-containing diet than in those fed the diet containing crystalline Fraction I protein from tobacco.

Organ weights. Organ weights of rats in the two dietary groups on the 29th day of the experiment are indicated in Table V. The weight of the liver, kidneys, spleen, thymus and seminal vesicles was significantly greater

RC Reference Casein, Sheffield Chemical Co., n, N.Y.

[4] Technicon Corporation, Tarrytown, N.Y.

TABLE II. COMPARATIVE EFFECTS OF CASEIN AND CRYSTALLINE FRACTION I PROTEIN FROM TOBACCO ON THE WEIGHT INCREMENT AND PER OF RATS (10 ANIMALS PER GROUP).

	Casein-containing diet (group I)	Fraction I protein-containing diet (group II)
Initial body weight[a]	48.8 ± 0.34	48.8 ± 0.56
Avg. weight increment (g) after the following days of feeding:[a]		
7 days	18.1 ± 0.80	25.7 ± 1.29[b]
14 days	51.5 ± 1.69	65.2 ± 1.87[b]
21 days	80.8 ± 2.40	100.6 ± 2.25[b]
28 days	114.4 ± 3.25	139.1 ± 2.78[b]
Avg. food consumption (g) after the following days of feeding:		
7 days	66.4	75.6
14 days	167.7	189.3
21 days	281.0	324.9
28 days	404.5	461.4
PER after the following days of feeding:[a]		
7 days	2.73 ± 0.07	3.40 ± 0.08[b]
14 days	3.07 ± 0.06	3.44 ± 0.03[b]
21 days	2.88 ± 0.06	3.10 ± 0.03[c]
28 days	2.83 ± 0.04	3.01 ± 0.02[b]

[a] Mean ± SE of the mean.
[b] Significantly different ($P < 0.001$) from group I value.
[c] Significantly different ($P < 0.01$) from group I value.

TABLE III. HEMATOLOGICAL FINDINGS OF RATS FED HIGHLY PURIFIED DIETS CONTAINING EITHER CASEIN OR FRACTION I PROTEIN FROM TOBACCO AS THE SOLE SOURCE OF PROTEIN (10 ANIMALS PER GROUP).

Hematological constituents	Casein-containing diet (group I)[a]	Fraction I protein-containing diet (group II)[a]
Hemoglobin, g/dl	14.1 ± 0.12	14.2 ± 0.09
Hematocrit, %	41.3 ± 0.67	42.3 ± 0.20
Red blood cells, millions per ml^3	6.62 ± 0.12	6.78 ± 0.07
White blood cells, thousands per ml^3	6.5 ± 0.66	5.7 ± 0.21
MCHC, %	34.5 ± 0.58	34.0 ± 0.12
MCH, mcmcg	20.8 ± 0.24	20.9 ± 0.17
MCV, cu. mic.	61.3 ± 0.78	61.3 ± 0.52
Polys, %	8.0 ± 1.19	7.2 ± 0.94
Lymph, %	92.0 ± 1.32	92.8 ± 1.03

[a] Mean ± SE of the mean.

in rats fed the diet containing crystalline Fraction I protein from tobacco than in rats fed the casein-containing diet. When the weight of these organs was expressed as weight per 100 g body weight, however, a difference between the two dietary groups was no longer manifest. No significant differences were observed between the two groups in heart, adrenal and testes weight. No differences were observed grossly in the appearance of the various organs between rats in the two dietary groups.

TABLE IV. SERUM LEVELS OF PROTEIN, LIPIDS AND OTHER CONSTITUENTS OF RATS FED PURIFIED DIETS CONTAINING CASEIN OR CRYSTALLINE FRACTION I PROTEIN FROM TOBACCO AS THE SOLE SOURCE OF PROTEIN (10 ANIMALS PER GROUP).

Serum constituents	Casein-containing diet (group I)[a]	Fraction I protein-containing diet (group II)[a]
Total protein, g/dl	5.7 ± 0.07	5.8 ± 0.07
Albumin, g/dl	4.5 ± 0.04	4.9 ± 0.06[b]
Globulin, g/dl	1.2 ± 0.05	0.9 ± 0.03[b]
A/G ratio	3.99 ± 0.17	5.53 ± 0.17[b]
Cholesterol, mg/dl	126 ± 3.72	121 ± 2.43
Triglyceride, mg/dl	55 ± 7.98	41 ± 6.22
Glucose, mg/dl	107 ± 20.88	85 ± 3.88
Urea nitrogen, mg/dl	9.3 ± 0.90	8.0 ± 0.53
Creatinine, mg/dl	0.5 ± 0.02	0.5 ± 0.02
Total bilirubin, mg/dl	0.2 ± 0.00	0.2 ± 0.01
Calcium, mg/dl	10.6 ± 0.10	11.0 ± 0.06[b]
Inorganic phosphorus, mg/dl	8.9 ± 0.36	8.7 ± 0.16
Sodium, mEq/L	139 ± 1.81	142 ± 0.93
Potassium, mEq/L	6.6 ± 0.21	5.8 ± 0.11[c]
Chloride, mEq/L	99 ± 0.60	99 ± 0.31
Carbon dioxide, mEq/L	23 ± 0.45	25 ± 0.20[b]

[a] Mean ± SE of the mean.
[b] Significantly different ($P < 0.001$) from group value.
[c] Significantly different ($P < 0.01$) from group I valu

Discussion. Present findings indicate tha tobacco which in the past has been grow primarily for smoking purposes can be proc essed to produce a water soluble crystallin

TABLE V. ORGAN WEIGHTS OF RATS FED HIGHLY PURIFIED DIETS CONTAINING EITHER CASEIN OR FRACTION I PROTEIN FROM TOBACCO AS THE SOLE SOURCE OF PROTEIN (10 ANIMALS PER GROUP).

	Casein-containing diet (group I)		Fraction I protein-containing diet (group II)	
Organ	Organ wt.[a]	Organ wt. per 100 g body wt.	Organ wt.[a]	Organ wt. per 100 g body wt.
Liver, g	5.61 ± 0.20	3.4	6.14 ± 0.09[b]	3.3
Kidneys, g	1.35 ± 0.05	0.8	1.58 ± 0.03[c]	0.8
Spleen, g	0.49 ± 0.02	0.3	0.57 ± 0.01[d]	0.3
Thymus, g	0.51 ± 0.03	0.3	0.62 ± 0.03[c]	0.3
Heart, g	0.72 ± 0.03	0.4	0.77 ± 0.02	0.4
Adrenals, mg	36.4 ± 1.4	22.3	41.4 ± 2.3	22.0
Testes, g	2.33 ± 0.05	1.4	2.40 ± 0.03	1.3
Seminal vesicles, g	0.33 ± 0.02	0.2	0.44 ± 0.02[d]	0.2

[a] Mean ± SE of the mean.
[b] Significantly different ($P < 0.05$) from group I value.
[c] Significantly different ($P < 0.01$) from group I value.
[d] Significantly different ($P < 0.001$) from group I value.

otein of high biological value. The latter rystalline Fraction I protein) is composed itirely of amino acids (6). In contrast to the raction I protein isolated from the leaves of her plant species which contain up to 5% rbohydrate (7), crystalline Fraction I pro- in from tobacco contains less than 0.0005% rbohydrate as measured by gas–liquid romatography-mass spectra analyses (6). he absorption spectra of crystalline Fraction protein from tobacco shows no evidence of cleic acids, visible pigments or materials hich absorb at wave lengths greater than 0 namometers (8). It contains no minerals ith the exception of the sulphur present in e sulphur-containing amino acids (9). In ew of the above and because of its high itritional value as a source of protein, it has e potential of being developed into impor- nt therapeutic products for the treatment of number of medical conditions. It would pear to be particularly valuable for the eding of patients with various types of renal sease in which sodium and/or potassium take must be rigorously controlled (10). nce it is readily soluble in water, it can be corporated in low residue nutritionally mplete liquid diets for the treatment of tients with pylorospasms, gastrointestinal stula, short bowel syndrome, tumors of the ophagus and gastrointestinal tract, pancrea- tis and other diseases involving maldigestion d malabsorption. Further studies are indi- ted to determine the effects of graded levels f crystalline Fraction I protein in the diet on ngth of survival and the incidence and se- verity of pathological changes associated with aging, its effect on reproductive and lactation performance, immunocompetence, resistance to stressor agents and possible toxic manifes- tations resulting from its long time ingestion in various species of experimental animals.

Summary. Weanling male rats were fed purified diets containing 10% protein in the form of either casein or twice crystallized Fraction I protein from tobacco. Findings indicated that the average weight increment and PER (protein efficiency ratio) of rats fed the diet containing Fraction I protein from tobacco were significantly greater throughout a 28-day experimental period than that of rats fed the diet containing casein. No signif- icant differences in hematological findings were noted between rats in the two dietary groups.

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'erential Effects of Cholera Toxin Pre-treatment on *In Vitro* vs. *In Vivo* Immunocyte Responses (40111)

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holera exoenterotoxin (CT) derived from os has marked immunoregulatory prop- s when administered together with an ;en into an experimental animal or when d to lymphoid cells immunized *in vitro*). Although CT is known to stimulate ylate cyclase activity and alter cyclic osine monophosphate (cAMP) levels in hoid as well as nonlymphoid tissues), the mechanism of immunologic mod- on induced by the toxin is unknown. In resent study the *in vitro* hemolytic anti- plaque response to sheep red blood cells BC) was found to be markedly enhanced n murine spleen cells were obtained from treated with CT on the same day of ire initiation or one day earlier, similar e enhancement of the immune response vo when mice were given CT and RBCs e same time (3, 5). Furthermore, similar ie effects observed *in vivo,* splenocytes mice treated several days earlier with were markedly impaired in their in vitro une responsiveness to the sheep RBCs In contrast, CT induced only enhanced ody responses when added to normal iocytes *in vitro*, either on the day of cul- initiation or several days earlier.

ethods and materials. Experimental ani-. Adult male Balb/c mice 8–10 weeks of and weighing 18 to 20 grams each, were ined from Cumberland View Farms, ton, TNN and housed in groups of 6 to plastic mouse cages and fed Purina se pellets and water *ad libitum.*

ntigen. Sheep erythrocytes were obtained lsever's solution from Becton-Dickinson pany, Cockeysville, Maryland. The s were washed with culture medium by ifugation and resuspended to a 0.1% entration containing approximately 2 × RBC per ml.

holera toxin. The toxin was obtained from H. Miller, National Institute of Allergy and Infectious Diseases, Bethesda, MD, and had been prepared by Dr. Richard Finkelstein, Microbiology Department, University of Texas, Southwestern Medical School, Dallas, TX. The lyophilized toxin was reconstituted with sterile water and diluted with phosphate buffered saline containing 0.1% human serum albumin to a concentration of 200 μg/ml. The toxin was stored at −70° in small aliquots and immediately before use diluted in pyrogen-free saline.

In Vitro culture. The Marbrook culture system for in vitro immunization of splenocytes was used essentially as described earlier (10, 11). In brief, 5×10^6 washed splenocytes from normal or toxin treated mice were cultured in 0.5 ml minimal essential medium (MEM) containing 10% fetal calf serum (Flow Laboratories, Rockville, MD) on a sterile dialysis membrane in the inner chamber of a Marbrook vessel (Bioresearch Glass Company, Vineland, NJ). Eleven to twelve milliliter MEM were placed in the outer reservoir chamber. Cholera toxin (0.1 μg) and erythrocytes (2×10^6 SRBC) were added to the inner chamber either on the day of culture initiation or, alternatively, toxin was added on day 0 and SRBC was added on day +2. Controls consisted of splenocytes incubated similarly but in the absence of either toxin and/or SRBC, or both. All cultures were prepared in duplicate or triplicate and incubated, without rocking, at 37° in a humidified atmosphere of 83% N_2, 10% CO_2 and 7% O_2 for 5–6 days. Viability was assessed by the standard trypan blue dye exclusion procedure and total cell number determined with a hemocytometer.

Determination of antibody forming cells. The localized hemolytic assay in agar gel as described initially by Jerne and associates (12) was used to enumerate 19S IgM plaque forming cells (PFCs) in the culture chambers. In brief, 0.1 ml of a spleen cell suspension

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was added to 2 ml Difco Noble agar containing 0.1 ml of a freshly washed 10% suspension of SRBC. The agar cell mixture was then quickly poured onto the surface of a previously prepared 60 mm diameter petri plate containing a base layer of agar. After 1 hr incubation at 37° the plates were treated with several ml of a 1:10 dilution of guinea pig complement and incubated further for 30–60 min at 37°. Zones of hemolysis appearing on the surface of the agar plate were considered due to high efficiency IgM PFCs. The numbers of such PFCs for 10^6 viable cells or per whole culture were determined and the average for two to three chambers in each group calculated.

Experimental results. Immunization of normal splenocytes in vitro with SRBC resulted in a marked antibody response as evident by the appearance of numerous PFCs. When spleen cells were obtained from mice pretreated with 1.0 μg cholera toxin at various times before sacrifice varying numbers of PFCs appeared for similar numbers of spleen cells plated (Table I). The number of PFCs for spleen cell suspensions from CT treated mice was directly related to the time of the toxin pretreatment of the mice prior to culture initiation. There was little difference in the total number or viability of spleen cells in the cultures prepared from mice pretreated with toxin as compared to control mice. However, spleen cells from mice given toxin shortly before sacrifice showed a marked enhancement of PFC formation (approximately two-fold) as compared to control cultures. Mice given toxin 1 day before sacrifice shov less consistent enhanced responsivene SRBC, but in general there were usually antibody producing cells for these ce compared to controls.

Mice given CT 2 days before sac showed a suppression of their splenic responsiveness, similar to the suppressio CT-pretreated mice challenged directly then tested for splenic PFCs (Table I). thermore, mice given CT 3 days before rifice showed an even greater suppressi their PFC response, similar to earlier r of *in vivo* studies in which CT-pretreated showed marked impairment of antibod sponsiveness (5). No significant diffe was observed for the number of "l ground" PFCs in splenocyte cultures bated without SRBC, regardless of wh the spleen cells were obtained from CT treated or normal mice (Table I). In add there was little difference in the rate o pearance of PFCs in cultures prepared normal vs. CT-pretreated mice (Tabl Although the magnitude of the peak resp differed, in all cases the peak numbe PFCs occurred on day +5 after immu tion.

To determine whether the toxin influe the immune response directly or becau a possible indirect effect not associated lymphoid cells *per se*, spleen cells from mal Balb/c mice were cultured in vitro SRBC, either with or without CT. Whe μg CT was added to normal cultures o day of *in vitro* immunization, there w

TABLE I. IN VITRO ANTIBODY RESPONSE OF SPLEEN CELLS FROM NORMAL AND CHOLERA TOXIN PRETREATED MICE.

Spleen cell source[a]	Antibody PFC response[b]				Backgrou
	Per 10^6 spleen cells	Percent of control	Per culture	Percent of control	PFC respo per cultu
Normal (controls)	822 ± 21	—	1603 ± 68	—	72 ± 15
CT-treated[c] day 0	1525 ± 210	186	2850 ± 360	178	79 ± 32
−1	1209 ± 56	147	2290 ± 216	143	64 ± 19
−2	448 ± 136	54	860 ± 130	54	74 ± 30
−3	273 ± 76	33	563 ± 198	35	68 ± 27
−7	734 ± 42	89	1350 ± 210	83	78 ± 24
−10	829 ± 38	108	1560 ± 220	97	74 ± 3(

[a] 5×10^6 spleen cells from indicated group cultured *in vitro*.

[b] Average number of PFCs (±SE) for 6–8 cultures per group 5 days after *in vitro* immunization with 2×10^6 RBCs; number of nucleated cells per culture averaged 1.8×10^6 viable cells ± 0.25×10^6 for all cultures at t assay.

[c] Groups of 5–10 donor mice injected iv with 1.0 μg CT on indicated day before culturing.

marked enhancement of the PFC response. This dose of CT had no effect on cell viability or number. Similar effects were noted with CT ranging from 0.01 to 0.5 μg. Higher doses caused cell death and lower doses had no effect on antibody formation. The enhancement of antibody formation was as great if not greater than that which occurred when splenocytes were obtained from mice treated with CT on the day of sacrifice or 1 day earlier (Table III). In order to determine whether pretreatment of spleen cells *in vitro* with toxin could depress the immune response as occurred with pretreated mice, normal mouse spleen cell cultures were treated with 0.01–0.5 μg toxin on day 0 and SRBC added 2 days later. PFCs were assayed 4 days after *in vitro* immunization (i.e., 6 days after culture initiation). As can be seen in Table III, a marked enhancement of the PFC response was evident for these cultures treated with 0.1 μg CT before immunization, similar to the enhancement occurring when the same dose of CT was added to cultures on the day of *in vitro* immunization. When the CT dose was varied from 0.01 to 0.5 μg similar effects occurred. Such enhancement contrasted markedly with the depressed PFC response of spleen cells obtained from mice treated 2–3 days earlier *in vivo* with toxin.

TABLE II. CYTOKINETICS OF APPEARANCE OF ANTIBODY FORMING CELLS TO SRBC BY SPLEEN CELLS FROM NORMAL MICE PRETREATED WITH CHOLERA TOXIN AFTER IMMUNIZATION IN VITRO.

Time in days after *in vitro* immunization[a]	PFC per culture[b]		
		CT-pretreated on day[c]	
	Normal	0	−3
0	11 ± 3	35 ± 5	10 ± 6
+2	89 ± 19	148 ± 42	38 ± 17
+3	310 ± 26	570 ± 68	86 ± 28
+4	810 ± 38	1715 ± 195	193 ± 18
+5	976 ± 48	1930 ± 240	224 ± 65
+8	478 ± 39	1130 ± 140	110 ± 27

[a] 5×10^6 spleen cells from normal or CT pretreated (1.0 μg) mice immunized *in vitro* with 2×10^6 SRBC and PFC responses determined on indicated days thereafter.
[b] Average number of PFC for 3–5 culture per group (±SE).

Coculture of 5×10^6 spleen cells from three day CT pretreated mice together with an equal number of spleen cells from normal mice did not affect the expected immune response. In a typical experiment 1705 ± 122 PFCs occurred per culture containing normal splenocytes alone and 372 ± 96 PFCs developed in cultures containing the same number of spleen cells from three day CT pretreated mice. When equal number of these cells were cocultured together the total number of PFCs was 2218 ± 1491, a number quite close to that expected from the sum of the expected number of PFCs in cultures of normal and CT pretreated splenocytes. Varying the ratio of spleen cells from normal vs CT-pretreated mice similarly resulted in the expected number of PFCs. Thus it did not appear likely that the spleen cell suspension from CT pretreated mice contained "suppressor" cells which could affect the expected antibody response of spleen cells from untreated mice.

Discussion. Cholera toxin is known to modulate immune responses, both *in vivo* and *in vitro* (1–6). Earlier studies in this laboratory showed that injection of toxin into Balb/c mice prior to immunization with sheep RBCs

TABLE III. IN VITRO ANTIBODY RESPONSES OF SPLEEN CELLS FROM NORMAL MICE TREATED WITH CHOLERA TOXIN.

Culture group	Day culture immunized with SRBC[a]	CT-treatment of culture[b]	Antibody PF Response[c]			
			Per 10^6 spleen cells	Percent of control	Per culture	Percent of control
A_1	Day 0	−	533 ± 109	—	1005 ± 170	—
A_2	0	+	3951 ± 477	741	7540 ± 622	750
B_1	Day +2	−	339 ± 37	—	528 ± 46	—
B_2	+2	+	1715 ± 379	506	2680 ± 498	508

[a] 5×10^6 normal mouse spleen cells cultured in vitro and immunized either on day of culture initiation or 2 days later with 2×10^6 SRBC.
[b] Indicated spleen cell cultures treated on day of culture initiation (day 0) with 0.1 μg CT.
[c] Average PFC (±SE) response for 5–8 cultures per group; group A_1 and A_2 tested 5 days after culture initiation and *in vitro* immunization while group B_1 and B_2 tested on day 6 after culture initiation (4 days after *in vitro* immunization).

resulted in markedly depressed antibody responses (3, 5). Toxin treatment at the time of immunization in vivo was found to enhance the immune response. These contrasting effects of CT according to the time of toxin administration relative to the day of challenge immunization was attributed to effects on adenylate cyclase, the enzyme which catalyses the conversion of adenosine diphosphate to cAMP. The increased antibody responsiveness in mice given CT simultaneously with antigen or one day earlier is thought to be related to increased nucleotide levels (5, 8). On the other hand, decreased antibody formation in mice given CT several days before antigen appeared to be related to lower levels of cAMP. CT also affects the nucleotide levels of mouse lymphoid cells treated in vitro. However, only increased levels occurred, without the decrease.

In the present study, the enhancement of PFC responsiveness by normal splenocytes treated in vitro with CT, may also be related to altered cyclase and cAMP levels. The failure of CT pretreatment *in vitro* to depress the antibody response of mouse splenocytes immunized with SRBC could be due to the failure of the toxin to influence certain target cells, other than lymphocytes, which may be important for *in vivo* induced immunosuppression. It is also possible that lymphocyte depletion induced by CT injection into mice may influence the *in vivo* but not *in vitro* response to SRBC. Earlier studies in several laboratories, including this one, have shown a temporary decrease in lymphocyte number within 2–5 days after CT injection. However, maximum depression was never greater than 30%, while the depression of antibody forming cell often reached 70–80%, indicating that lymphopenia per se could not account for the observed immunologic impairment *in vivo*. However, CT appeared to have a preferential depressive effect on T cell number, since T dependent areas in the spleen were often affected after CT injection. Other studies in this laboratory, using immunofluorescent procedures, indicated a moderate depletion of lymphoid cells rich in theta antigen on their surface (presumably T cells) as well as cells with surface Ig globulins, presumably B cells. Depletion of lymphocytes *in vivo* may not directly be responsible for immunosuppression. Even thymic lymphopenia may not be a direct effect of the toxin. For example, it has been reported that observed depletion of lymphoid cells after CT treatment can be prevented by prior adrenalectomy of mice (13). Exogenous and endogenous adrenal glucocorticosteroid hormones are known to induce lymphocytopenia (13, 14). Stimulation of adrenal corticosteroid synthesis and release of corticotropin (ACTH) are influenced by cAMP (14). Thus it is possible that CT injection into mice causes an increase in the level of circulating adrenal cortical hormones which, in turn, produces an effect on lymphoid cells.

A marked dysfunction of lymphoid cells, even without their obstruction, may occur in terms of immune responsiveness. Even when similar numbers of spleen cells from normal and CT-pretreated mice are cultured with SRBC, the splenocytes from the toxin treated mice were found not to respond in a normal manner to the antigen. On the other hand, treating cells entirely in vitro with CT caused only an immunoenhancement when toxin was added 2 days before antigen to simulate as closely as possible the *in vivo* experiments which resulted in the immunosuppression. No impairment of immune responsiveness was evident in such *in vitro* treated cultures, as occurred when CT was given to mice 2–3 days before culture. Thus it seems likely that CT affects the immune response mechanism in a negative manner only when given *in vivo* so target cells other than those directly involved in antibody formation are influenced.

Studies in progress are directed at determining the nature of such possible target cells. It does not appear likely that CT induces or increases the number of, or activity of "suppressor" cells, since cocultivation of graded numbers of spleen cells from 3-day CT pretreated mice with equal numbers of spleen cells from normal mice did not influence the antibody response to SRBC *in vitro* (unpublished observations). The enhanced antibody response of spleen cells from mice given CT the day of sacrifice or by spleen cells treated *in vitro* with CT on the day of *in vitro* immunization or 2 days earlier, did not appear due to stimulation of helper or nonspecific enhancing lymphoid cells, since cocultivation experiments with normal spleen

in vitro did not result in PFC responses ;reater than would be expected from the ɔf the cell population used (unpublished ·vations).

mmary. Cholera toxin is known to affect ody responsiveness, both *in vivo* and *in* In the present study spleen cells from pretreated with cholera toxin were edly deficient in their ability to respond ɛep red blood cells by forming hemolytic odies in vitro, whereas splenocytes from treated with toxin on the day of immuion or 1 day earlier, showed marked ncement of antibody responsiveness *in vitro* challenge immunization. Spleen from normal mice treated *in vitro* with ;ra toxin at the same time as immunizaalso showed a marked enhancement of mmune response. Furthermore, normal n cell cultures first treated with cholera *in vitro* and then immunized 2 days with sheep erythrocytes showed an ined rather than decreased PFC response. e results indicate that depression of any formation observed *in vivo* or *in vitro* spleen cells from cholera toxin pre-ɛd animals may reflect an indirect effect, eas increased responses induced by toxin sure on the day of immunization, either *in vivo* or *in vitro,* may reflect a direct enhancing effect on immunocytes *per se.*

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Live Respiratory Syncytial Virus Vaccine Administered Parenterally (40112)

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Respiratory syncytial virus, a member of the Paramyxoviridae, Genus Pneumovirus, (1), is recognized as the single most important viral agent causing serious respiratory disease in infants and young children (1–5). The virus is an important cause for bronchitis, bronchopneumonia, and pneumonitis in young children and is a frequent cause of coryzal illness in older children and adults in whom lower respiratory tract infection may also occur. First infection with the virus, commonly associated with bronchiolitis and pneumonia, often occurs in early life and is sometimes fatal.

There is no currently acceptable vaccine against respiratory syncytial virus infections. Killed virus vaccines have not presented evidence of inducing high-level immunity (6–10). Live attenuated virus vaccines (11–15) given directly into the respiratory tract have not been satisfactorily developed to date.

The present report describes the development and clinical testing of a new and unique live attenuated respiratory syncytial virus vaccine that is administered by parenteral injection. Infants and young children who are initially without neutralizing antibody against the virus develop such antibody following vaccination. The vaccine causes minimal, if any, clinical reaction.

Materials and methods. Vaccine. A throat swab taken January 26, 1973 from a patient (S.A., C-73-287) with respiratory syncytial virus infection was obtained on May 23, 1973 from Dr. Robert Chanock. An isolate, identified as strain 287 respiratory syncytial virus, was recovered in cell cultures of grivet monkey kidney that were prescreened to rule out presence of adventitious agents. One additional passage was made in grivet monkey kidney cells and subsequent passages were made in WI-38 line diploid human embryonic lung cell cultures that were monitored in accordance with the "Revised Standards for Karyology of Human Diploid Cell Cultures" (16). Virus that was propagated in fifth and in tenth WI-38 cell culture passage was used to prepare Lots 594 and 592, respectively, of vaccine. Virus-infected cell culture fluids were clarified by passing through a medium (15 micron) sintered glass filter, dispensed in 1.2 ml amounts into vials, and lyophilized. Appropriate *in vivo* and *in vitro* tests, including tests in monkeys, were carried out on the pre-clarified virus samples, on the vaccine, and on uninoculated control culture fluids to rule out the presence of extraneous microbial and viral contaminants by procedures that are consistent with current standards for safety of live virus vaccines (17). Electron microscopic examination of the vaccine using a thin sectioning technique (18) revealed 10^7–10^8 viral particles per ml. There was insufficient viral antigen in the vaccine to permit detection in the complement–fixation test (1:2). *Titrations* of viral infectivity were carried out in Hep-2 cells read 14 days after inoculation. The infectivity titer of passage 5, Lot 594, vaccine was $10^{-2.5}$/0.1 ml and that for passage 10, Lot 592 vaccine was $10^{-2.6}$/0.1 ml. *Serum neutralization* tests were performed using Hep-2 cell cultures. Strain 287 of respiratory syncytial virus seed stock was diluted to contain 10–30 $TCID_{50}$ per 0.1 ml and was mixed with equal volumes of serial twofold dilutions of subject sera that were inactivated by heating at 56°C for 30 min. Duplicate cell cultures were inoculated with 0.2 ml of the mixtures following incubation at 37°C for hr and incubated at 37°C for 7 days at which time observations were made for cytopathology. The serum titer was the highest initial dilution of serum that totally prevented viral cytopathology. Titrations of the viral seed stock were carried out simultaneously to establish the $TCID_{50}$ dose. Cytopathology first

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d on the fifth day in cultures inocu-
th 10–30 $TCID_{50}$ of virus.

al populations. Two studies were car-
among infants and children from
class socioeconomic background who
good health and who resided in open
ons in the Havertown area of subur-
ladelphia. The studies were done in
cians' offices and informed written
were obtained from the parents or
is. *Study 487* of Lot 594 (passage 5)
was carried out between October 12,
d April 26, 1977 among 39 infants
ldren 9 months to 4 years of age.
one participants received 0.5 ml of
subcutaneously and 18 older siblings
contacts served as unvaccinated con-
trols. *Study 456* of Lot 592 (passage
ine was carried out between April 7
y 19, 1976 among 27 infants and
, 6 months to 3 years of age. Fifteen
ants received 0.5 ml of vaccine sub-
usly. Twelve older siblings served as
controls. Temperatures were taken
/ the mothers for 28 days, and all
were reported to one of us (REW).
made telephone calls twice a week
ome visit on day 5 or 6 to observe all
and encourage close observation, re-
and reporting by the parent. Any
ho became ill was cared for by his
n. Blood samples were taken from all
lren immediately prior to and 6 weeks
vaccine was given in order to mea-
ibody responses to the vaccine and
ossible spread of infection to suscep-
itact controls. Nasal washings, free of
vere collected from a portion of the
in Study 487 to test for nasal anti-
sponse following vaccination. The
were taken in 5 ml of saline solution
nasally.

*ts. Neutralizing antibody responses to
ion in initially seronegative children.*
shows the homologous antibody titers
s after vaccination among children
eived passage 5 or 10 respiratory syn-
rus vaccine or who served as unvac-
controls. Ninety-three percent of pas-
nd 75% of passage 10 vaccine recipi-
veloped antibody following vaccina-
d none of the 11 contact controls
ed such antibody. Two of six persons tested who received passage 5 vaccine displayed detectable neutralizing antibody in the nasal secretions and none of the three initially seronegative contact controls developed antibody. The protein content of the nasal secretions ranged from 7.8 to 104.0 mg/100 ml. Not all secretions contained sufficient protein to permit detection of antibody, but the protein content of the two samples from each of the two patients in whom responses were noted were roughly the same (patient 25, 73.2 and 104.0 mg; patient 37, 36.4 and 49.7 mg).

Tests for neutralizing antibody responses in 14 initially seropositive children given the vaccine. As shown in Table II, 2 initially seropositive children (Nos. 18 and 24) showed fourfold or greater increases in antibody following vaccination, three were indeterminate, and nine showed no change.

Clinical reactions among the subjects. As might be expected, many of the children displayed episodes of respiratory illness during the 28-day observation period following vaccination. Table III summarizes the composition of the test and control groups according to age and number, and the numbers of respiratory disease episodes that were observed. All illnesses were mild, limited to the upper respiratory tract, and were diagnosed as colds or pharyngitis. No child displayed lower respiratory tract involvement. The distribution of such illnesses was not remarkably different between the groups whether initially seronegative, seropositive, and whether given vaccine or not. There was no indication of illness caused by the vaccine. If, indeed, such did occur it would have been very mild and clinically inconsequential.

Table IV shows the occurrence of fever, according to time after vaccination, among the initially seronegative and seropositive children who received the vaccine and among the unvaccinated controls. The occurrence of fever appeared to be randomly distributed among all the groups. Fever caused by the vaccine was infrequent, if it occurred at all.

Distribution of antibody, according to age. All the initial serum samples taken from the 66 subjects in the study were tested for presence and amount of neutralizing antibody against respiratory syncytial virus with the findings shown in Fig. 1. Among the subjects, there was gradual increase in percentage of

TABLE I. HOMOLOGOUS NEUTRALIZING ANTIBODY RESPONSES IN YOUNG INITIALLY SERONEGATIVE CHILDREN GIVEN LIVE RESPIRATORY SYNCYTIAL VIRUS VACCINE BY THE SUBCUTANEOUS ROUTE.

Vaccine		Patients			Neutralizing antibody response: Circulating			Nasal
						Seroconversion		
Lot No.	Passage No.	Group	Ident. No.	Age (Mos.)	Titer	No. Total	Rate	Titer
594 (C-D495)	5	*Vaccine*				13/14	93%	
			7	9	<2			
(Study 487)			31	9	4			<2
			24	10	4			
			25	10	4			2
			27	13	4			<2
			36	13	8			<2
			1	14	2			
			19	14	2			
			11	15	8			
			37	15	16			4
			5	16	4			
			18	18	32			
			13	20	2			
			22	32	16			<2
					Mean (4.9)			
		Control				0/8	0%	
			35, 30, 32, 20, 2, 12, 17, 8	16, 20, 20, 25, 28, 31, 43, 46	<2 <2 <2			<2, <2, <2
592 (C-D478)	10	*Vaccine*				6/8	75%	
			4	7	<2			
(Study 456)			13	7	2			
			8	11	<2			
			20	11	16			
			22	12	2			
			12	15	16			
			23	18	8			
			14	19	2			
					Mean (3.4)			
		Control						
			3, 7, 11	22, 28, 33	<2	0/3	0%	

seropositives with increasing age. Of the total group of persons, who were in the 6 to 48 months of age range, half were seronegative.

Discussion. The frequently severe and sometimes fatal respiratory illnesses in young children caused by respiratory syncytial virus infection justifies concerted effort to develop an effective vaccine. Reinfection that occurs in older children and adults is usually far less severe than in infants and manifestations of the illness are ordinarily limited to the upper respiratory tract, though bronchitis, bronchopneumonia or pneumonia may occur (1, 2, 11). The fact that frequency and severity of illness is inversely related to presence of circulating homologous neutralizing antibody (19, 21, 22) provides a basis for preventing or modifying the natural disease by vaccines.

Killed respiratory syncytial virus vaccines though capable of stimulating antibody, have not shown great promise for preventing infection or illness caused by the virus. Infants given one particular killed vaccine (8–10) developed more serious illness than those who were not vaccinated. In attempts to explain this phenomenon, Chanock and co-workers (20) suggested that an immunopathologic reaction might be involved in the lung of the children, on natural infection, that is due to absence of IgA antibody and the presence of circulating neutralizing antibody of the IgG class induced by killed vaccine or

ively transferred from the mother. Va- y for this hypothesis is doubtful since nt studies have shown a relative sparing ct (21) from the disease in children less 1 1 month of age when maternally ac- ed IgG antibody is at its highest level and e the severity of pneumonias caused by iratory syncytial virus in early infancy is rsely related to the level of circulating tralizing antibody (19, 21, 22). The more re illness that occurred in infants who e given killed virus vaccine might have 1 due to the relative impurity of the vac- used that might have induced sensitiza-

BLE II. NEUTRALIZING ANTIBODY FINDINGS IN NITIALLY SEROPOSITIVE CHILDREN WHO WERE VACCINATED.

'accine	Subject No.	Serum neutralizing antibody titer: Before vaccination	Serum neutralizing antibody titer: After vaccination
594	3	16	16
. 5 vac-	28	32	32
ne)	9	64	64
	15	64	64
	29	≥64	128
	33	≥64	128
	38	≥64	256
592	16	2	2
. 10 vac-	10	32	32
ne)	18	64	256
	6	128	128
	24	128	1024
	26	128	128
	2	1024	1024

tion to the tissue components in the vaccine or to tissue–viral antigen combinations.

Two live respiratory virus vaccines developed by Chanock *et al.* (11, 12, 15) that are administered by nasal inoculation have been tested in man. *RS-A2* (RSV-26°) vaccine (11, 13, 15), made from virus adapted to growth at low temperature, displayed limited infectivity for adults, was poorly immunogenic in infants and young children who were having first experience with the virus, and was not considered suitable for vaccination purpose. A second vaccine, prepared from a temperature-sensitive mutant of respiratory syncytial virus (ts-1) (12, 13, 14), was also considered unsuitable for routine use in young children because it produced illness in susceptible individuals, produced infections in which virus was shed that were contagious to susceptible contacts, and showed partial genetic reversion to "wild-type" genotype (23) following human infection. All of a group of initially seronegative children less than 6 years of age and about half of children 6–13 years of age developed neutralizing antibody. There was no evidence for potentiation of natural illness by the live attenuated virus vaccine (14).

The respiratory syncytial virus vaccine described in this report is novel in that it was administered parenterally and used the same route of administration as for live measles, mumps, and rubella virus vaccines. Most importantly, 19 of the 22 initially seronegative individuals (86%) developed circulating antibody and 2 of 6 of these persons who were tested developed detectable amounts of anti-

LE III. RESPIRATORY DISEASE EPISODES,[a] ACCORDING TO INITIAL SEROSTATUS AMONG VACCINEES AND CONTROLS.

Test group	Day of onset of illness	Pas. 5 vaccine (Lot 594): No. in group (age in mos.)	Pas. 5 vaccine (Lot 594): No. with illness (%)	Pas. 10 vaccine (Lot 592): No. in group (age in mos.)	Pas. 10 vaccine (Lot 592): No. with illness (%)
Vaccinated					
Initial seronegative	0–2	14 (9–32)	3 (21)	8 (7–19)	2 (25)
	3–14		6 (43)		4 (50)
	15–21		5 (36)		2 (25)
Initial seropositive	0–2	7 (9–21)	0 (0)	7 (6–19)	1 (14)
	3–14		5 (71)		2 (29)
	15–21		2 (29)		1 (14)
Unvaccinated controls					
All persons	0–2	18 (16–48)	2 (11)	12 (22–44)	1 (9)
	3–14		7 (39)		1 (9)
	15–21		5 (28)		1 (9)

[a] All were cases of upper respiratory illness.

TABLE IV. MAXIMUM TEMPERATURE, ORAL (°F), ACCORDING TO INITIAL SEROSTATUS, AMONG VACCINEES AND CONTROLS (SEE TABLE III).

Test group	Time period after vaccination (days)	Maximum temperature—No. (%)						
		Vaccine pas. 5 (Lot 594)				Vaccine pas. 10 (Lot 592)		
		<99	99–100.9	101–102.4	104.0	<99	99–100.9	101–102.0
Vaccinated								
Initial seronegative	0–2	4 (28.6)	9 (64.3)	1 (7.1)		3 (37.5)	5 (62.5)	
	3–14	4 (28.6)	8 (57.1)	2 (14.3)		1 (12.5)	6 (75.0)	1 (12.5)
	15–21	5 (35.7)	8 (57.1)	1 (7.1)		5 (62.5)	3 (37.5)	
Initial seropositive	0–2	4 (57.1)	3 (42.9)			3 (42.9)	4 (57.1)	
	3–14	4 (57.1)	2 (28.6)	1 (14.3)		2 (28.6)	5 (71.4)	
	15–21	5 (71.4)	1 (14.3)	1 (14.3)		2 (28.6)	5 (71.4)	
Unvaccinated controls								
All persons	0–2	11 (61.1)	6 (33.3)	1 (5.6)		3 (25.0)	9 (75.0)	
	3–14	7 (38.9)	9 (50.0)	2 (11.1)		2 (16.7)	10 (83.3)	
	15–21	7 (38.9)	10 (55.6)		1 (5.6)	7 (58.3)	4 (33.3)	1 (8.3)

body in the nasal secretions as well. As with mumps vaccine (24), the circulating neutralizing antibody titers obtained were far less (mumps mean about 8) than found following natural infection (mumps mean about 40), and few persons with preexisting antibody showed antibody increase on vaccination. Though respiratory syncytial virus is not ordinarily associated with systemic infection and viremia, there appeared to be sufficient replication following parenteral injection to permit development of a substantial circulating antibody response that was also found in the nasal secretions. Importantly, none of the vaccinated susceptible persons showed more than very minor respiratory illness, if any at all. None of the susceptible contacts developed antibody against the virus, indicating lack of contagious spread from the vaccinated persons. The vaccine, as presently constituted, presents very desirable attributes for a live virus vaccine and warrants continued studies to determine the efficacy in preventing illness caused by this agent. Importantly, the vaccinated subjects are being followed for clinical response on reinfection in nature with respiratory syncytial virus.

The question *might* reasonably be asked whether there was actual replication of the virus in the human subjects or whether the antibody response was due to presence of nonreplicating viral substance in the vaccine. Viral replication appears highly likely because of the extremely small quantity of virus in the vaccine (10^7–10^8 particles per ml and less-than-detectable amount of complement-

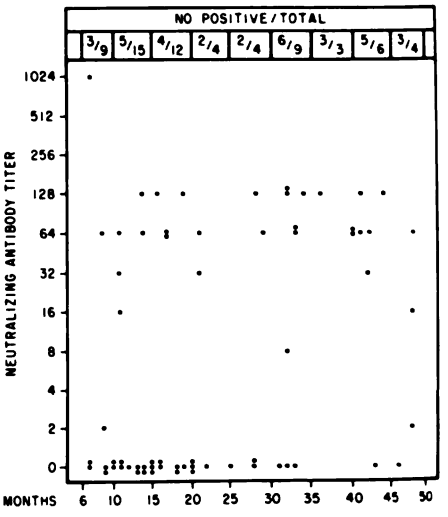

FIG. 1. Distribution in children, according to age, o neutralizing antibody against respiratory syncytial virus

fixing antigen), about 100-fold smaller in amount than the antigen mass needed, in our experience, to induce antibody in man for other viruses. For example, about 10^{11} particles of killed influenza viral antigen are needed per dose. Attempts, to date, not reported here, to isolate virus from the respiratory secretions of human subjects have also been negative. Respiratory secretions taken from 16 susceptible individuals at 3–5 day intervals for 21 days after vaccination were free of detectable respiratory syncytial virus. This is not surprising since virus also cannot be recovered from the respiratory secretions of subjects given measles or mumps vaccine

to detect viremia in vaccinated sub-
e not been carried out to date since
tion does not appear to justify the
repeat venipunctures in young chil-

uestion might also be asked as to
sons might benefit most by use of
ratory syncytial virus vaccine. Clues
ided by data relating to the natural
ce with the virus. Chanock *et al.* (25)
it essentially all infants are born with
utralizing antibody of maternal ori-
hat such antibody decreases in titer
nately twofold per month. First in-
vith respiratory syncytial virus typi-
urs within the first few years of life.
nity-wide epidemics of the infection
ur every year during the fall, winter,
ng seasons (25). As noted above,
ing antibody that arises following
is of long-term persistence and is
d with illness of reduced severity
nfection occurs. Antibody of mater-
n appears to afford substantial pro-
n early life, and this is substituted by
acquired antibody that is reinforced
ted infections thereafter. Reinfection
es, in spite of circulating antibody, is
on event, e.g., poliomyelitis, measles,
and rubella. Infections that depend,
s, upon systemic distribution are usu-
cally inapparent on reinfection (mea-
nps, rubella, poliomyelitis) while re-
ctions that are more superficial (in-
respiratory syncytial virus) com-
ause illness, though less severe.
uld be desirable to acquire active
y against respiratory syncytial virus
cy and early childhood by use of a
that causes no apparent clinical re-
n the open populations investigated
present study, only half of the 66
6–48 months of age, had demonstra-
body against the virus. In such a
on, the vaccine might have special
ss during early infancy and in the
l years as well. Immunization in the
stnatal period, before 6 months of
uld be complicated by the unpredict-
sence in some individuals of neutral-
tibody that suppresses an active im-
sponse. Administration of more than
dose of the vaccine might be required
n maximal benefit from use of the
vaccine. The importance of the disease caused by respiratory syncytial virus, the deficiency of antibody among children who might benefit from vaccination, and the ability of the parenterally administered live virus vaccine to induce antibody without causing illness justify further study of the present vaccine to measure its protective efficacy and the optimal conditions for its use.

Respiratory syncytial virus is a member of the family Paramyxoviridae (1) that includes the genera Paramyxovirus (paramyxoviruses and mumps), Morbillivirus (measles–distemper), and Pneumovirus (respiratory syncytial virus). Parenterally administered live measles and mumps virus vaccines are highly satisfactory and are routinely administered. The present respiratory syncytial virus vaccine represents a third example of a vaccine of the family Paramyxoviridae given parenterally. In further studies (unpublished), we have employed the same approach to parenteral administration of live parainfluenza 1, 2, and 3 virus vaccines. Types 2 and 3 have shown considerable promise in human trials to date. Parainfluenza 1 virus has been of less promise to date, since it grows only poorly in any presently known cell culture medium suitable for use in man.

Summary. A unique live attenuated respiratory syncytial virus vaccine that is administered parenterally is described. Nineteen of 22 initially seronegative children, 7–32 months of age, developed homologous circulating neutralizing antibody against the virus and a portion were shown to have present demonstrable antibody in their nasal secretions. None of the children developed illness that could be ascribed to the vaccine, and there was no evident contagious spread of the vaccine virus to susceptible contacts. The vaccine is of considerable promise and trials to determine protective efficacy are being pursued. Seroepidemiologic investigations revealed that only 33 of 66 children 6–48 months of age and residing in an open population had detectable circulating neutralizing antibody against the virus.

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Inhibition of Insulin and Glucagon Secretion by Somatostatin: are there Indirect Effects?[1] (40113)

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natostatin, a peptide isolated originally the hypothalamus, is known to inhibit ecretion of insulin and glucagon (1). this effect can be demonstrated *in vitro*, been concluded that somatostatin acts ly upon the pancreatic A- and B-cells . Further, immunocytochemical studies shown the presence of somatostatin-like ial within islet D-cells suggesting that it a role in the local regulation of endo- pancreatic function (4, 5). Recent inves- ons by this laboratory showed that al- drenergic blockade with phentolamine edly reduced the inhibitory action of ostatin upon insulin and glucagon e from the pancreas of dogs (6–8). observations raise the possibility that ostatin also affects islet function indi- by interacting with neural signals to the rine pancreas. In the present study, so- tatin was infused at various doses and ferent anatomic sites into anesthetized to determine whether an inhibitory ac- f this peptide upon insulin and glucagon ion could be found via specific routes a dose that was not effective when given ly into the pancreas. Our results indi- hat somatostatin may inhibit the release ulin and glucagon by indirect as well as mechanisms.

terial and methods. The surgical proce- used in these experiments has been de- d previously (9). Briefly, mongrel dogs (25–35 kg) were anesthetized with morphine sulfate and sodium pentobarbital following an overnight fast. A midline laparotomy was performed, and the blood flow of the superior pancreaticoduodenal vein (PDV) was shunted through an extracorporeal flow-meter circuit before reaching the portal vein. Blood samples were collected from this circuit for the determination of plasma glucose, immunoreactive insulin (IRI), and immunoreactive glucagon (IRG) using methods reported previously (9, 10). Measurement of PDV blood flow and hematocrit in addition to plasma hormone concentrations in the PDV enabled the calculation of IRI and IRG output from the pancreas (9). Throughout these experiments, rectal temperature was maintained (38.5 ± 0.5°) by using an autoregulated thermal blanket. Tracheal intubation and artificial respiration permitted maintenance of arterial blood gases within the normal range (pO_2 80–90; pCO_2 30–40; pH 7.37–7.0).

Dihydrosomatastatin (a gift from Dr. Roger Guillemin, Salk Institute) was infused into the superior pancreatic artery at rates of 0.17, 1.7, or 17 μg for 3 min. In other experiments, somatostatin was administered at a rate of 1.7 μg/min for 30 min into either the hepatic portal vein or a femoral vein. Blood samples were collected at −30, −15, −5, and 0 min prior to the infusions, at +5, +10, +20, and +30 min during somatostatin infusion, and thereafter at +35, +40, +50, and +[illegible]0 min.

Statistical evaluation of the data was performed by correlation analysis and Student's paired *t* test for IRI and IRG outputs, and analysis of variance for plasma glucose and PDV blood flow.

Results. Regardless of the site of infusion, somatostatin produced a decline of IRI (range, 217–21,874 μU/min) and IRG (range,

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1509–24,577 pg/min) output that was correlated linearly with the basal secretory rates of these hormones ($r = 0.88$ and 0.79, respectively, both $P < 0.001$, $n = 20$). Because of this consistent relationship, IRI and IRG output during all somatostatin infusions were calculated as the percent change (%Δ) of the basal rates of secretion. Figure 1 shows the temporal responses of plasma glucose levels and the outputs of IRI and IRG during an infusion of somatostatin (1.7 μg/min for 30 min) via the pancreatic artery.

The dose-response relationship between log increases of somatostatin dose infused via the pancreatic artery and the %Δ of IRI and IRG outputs are summarized in Fig. 2. At a rate of 0.17 μg/min ($n = 5$) somatostatin did

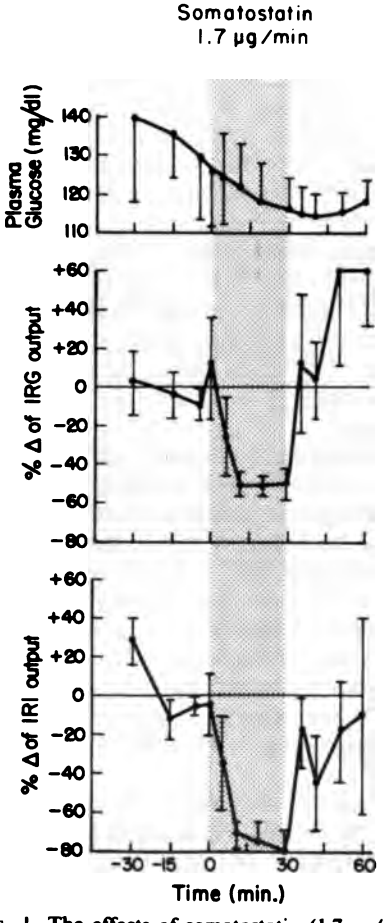


FIG. 1. The effects of somatostatin (1.7 μg/min for 30 min) upon plasma glucose level, IRI, and IRG outputs during an infusion via the pancreatic artery of dogs. Data are shown as mean ± SEM.

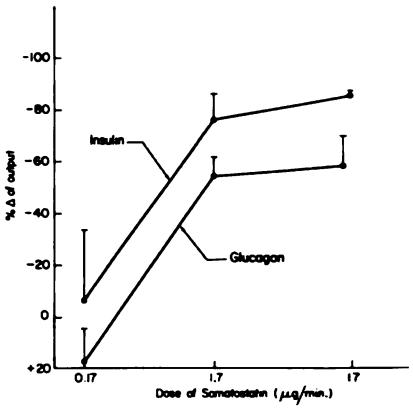
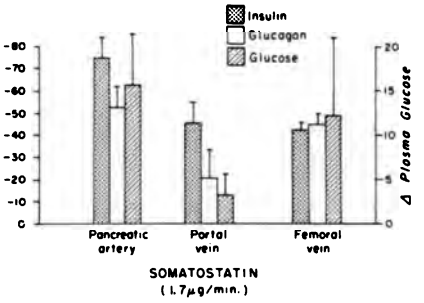


FIG. 2. Relationship between log dose of statin and the %Δ of basal IRI and IRG outpu tostatin infused via pancreatic artery. Values the mean of the 10, 20, and 30 min %Δ of e Mean ± SEM of each data point is given in tex

not reliably alter the basal output of hormone. When the infusion rate w creased to 1.7 μg/min ($n = 4$), IRI decreased markedly (%Δ = −76 ± 9, ± SEM, $P < 0.01$) as did IRG output −53 ± 8, $P < 0.01$). Increase of the s statin infusion rate to 17 μg/min (n resulted in an inhibition of the outputs (%Δ = −85 ± 3, $P < 0.001$) and IRG −57 ± 12, $P < 0.05$) that was not signifi different from that observed during th ceding dose of somatostatin. Thus, it appear that the inhibition of both IR IRG outputs was approaching maximu ing an infusion of somatostatin at 1.7 μ

To evaluate further its inhibitory upon the endocrine pancreas, somat was infused into either the pancreatic the hepatic portal vein, or a femoral v a rate of 1.7 μg/min for 30 min. Fi summarizes the findings of this stu indicated above, an infusion of somat at 1.7 μg/min via the pancreatic arter duced a significant inhibition of both II IRG outputs. This infusion also dec the level of plasma glucose ($P < 0.05$). infused into the hepatic portal vein, thi of somatostatin elicited a significant de of IRI output (%Δ = −46 ± 12, n = 0.05) while the apparent decline of IRG basal levels was not significant (%Δ ± 12). Plasma glucose levels also dec ($P < 0.05$). An identical infusion into



. 3. Comparison of the effects upon IRI and IRG ls and plasma glucose of infusions of somatostatin g/min for 30 min) administered via pancreatic , portal vein, or femoral vein. Data are shown as ± SEM.

vein ($n = 4$) elicited a decrease of IRI ut ($\%\Delta = -44 \pm 11$, $P < 0.02$) and a arable decrease of IRG output ($\%\Delta = \pm 5$, $P < 0.001$). Although there was a decline of plasma glucose levels during omatostatin infusion, the trend was not tically significant. No statistically signif- alterations of PDV blood flow were ved during any infusion of somatostatin.

scussion. Numerous investigations using *ro* preparations have shown that soma- tin is a potent inhibitor of insulin and gon secretion (1–3, 11). Those reports led, therefore, to the widely accepted pt that this peptide acts directly upon ancreatic endocrine cells. Similar con- ns have been drawn from the effect of tostatin *in vivo* due to its rapid inhibition ncreatic endocrine function (12–14). In tudy, infusions of somatostatin into the reatic artery of anesthetized dogs ptly decreased both IRI and IRG out- an effect that was reversed rapidly at ermination of the infusions. Thus, our nt results are in basic agreement with onclusion that somatostatin affects the A- and B-cells directly. Nevertheless, our vation of decrements of IRI and IRG uts when somatostatin was given via a ral vein or the hepatic portal vein sug- that, in addition to a direct effect upon ndocrine pancreas, somatostatin may in- islet hormone release through an indi- nechanism as well.

ood flow to the canine pancreas is ap- imately 2% of total cardiac output (15). efore, we expected that when somato- statin at a dose of 1.7 μg/min was infused via a femoral vein or the hepatic portal vein, only 2% of that amount would reach the pancreas (i.e., approximately 0.034 μg/min). Since a dose of 0.17 μg/min given directly into the pancreatic artery had failed to decrease IRI and IRG outputs significantly, no inhibitory effect was expected following infusions of somatostatin at 1.7 μg/min via the other sites. However, when the above mentioned dose of somatostatin was given into a femoral vein, both IRI and IRG outputs were inhibited significantly. An identical infusion into the hepatic portal vein also elicited a significant decrement of IRI output that was comparable to the effect observed when somatostatin was administered via a femoral vein. Therefore, peripheral and portal infusions of somato- statin were more potent inhibitors of insulin secretion than could be explained by reason- able blood flow calculations. It is difficult to draw conclusions regarding the intraportal action of somatostatin on IRG output, since the effects of this infusion were to produce a decrease of glucagon secretion that was nei- ther significantly different from basal levels nor significantly different from the decrease observed by infusions of somatostatin into a femoral vein.

The unexpected inhibition of IRI and IRG outputs during peripheral infusions of soma- tostatin and the effect of this peptide on IRI output when administered intraportally do not seem consistent with potential artifacts such as a somatostatin-mediated decrease of pancreatic blood flow or the reflux of this peptide into the pancreas. Although it has been reported that administration of soma- tostatin to humans reduced splanchnic blood flow (16, 17) leading to a potential decrease of islet hormone output, no statistically sig- nificant change of pancreaticoduodenal vein blood flow was observed during the admin- istration of this peptide to dogs. Reflux of somatostatin into the pancreas during infu- sions into the hepatic portal vein seem un- likely, since the monitored blood flow was always in the other direction. Further, this type of artifact cannot explain the effective- ness of somatostatin when given via a femoral vein. Thus, because the concentration of so- matostatin reaching the pancreas during in- fusions into a femoral vein or the hepatic

portal vein is presumably far less than that required to elicit a direct inhibitory effect, it seems reasonable to postulate that this peptide also alters islet hormone secretion by as yet unknown mechanisms.

Although the precise nature of this presumed indirect effect of somatostatin upon the endocrine pancreas is unclear, several explanations could account for the inhibition observed during this study. It is possible that, during a peripheral infusion, dihydrosomatostatin is converted to a more potent form. Preliminary work in this laboratory suggests that, when administered via a femoral vein, cyclic somatostatin is a more potent inhibitor of insulin secretion than dihydrosomatostatin, but that both forms of somatostatin exert comparable inhibitory effects upon glucagon secretion. However, previous studies by others show that dihydrosomatostatin and the oxidized or cyclic somatostatin are equipotent as inhibitors of insulin and glucagon release both *in vivo* and *in vitro* (18, 19). Conversion to an analog of somatostatin seems extremely unlikely due to the chemical changes required. Further, somatostatin analogs generally exhibit less biologic activity than the native peptide in terms of their inhibition of insulin and glucagon secretion (20, 21). Another factor that argues against a change of peptide form is the rapid metabolic clearance of somatostatin from the general circulation (22).

As alternative explanations for the presumed indirect action of somatostatin upon IRI and IRG output, we hypothesize that this effect may be related to a decrease of endogenous stimulatory signals for islet hormone release or to the initiation of an inhibitory neural-pancreatic reflex. Several studies have shown recently that somatostatin suppresses the circulating levels of gastrointestinal hormones including gastrin (1), secretin (23), and gastric inhibitory polypeptide (24). Since several intestinal hormones are known to potentiate the release of insulin and glucagon (25, 26), it seems reasonable to postulate that the present results may have been due to a decrease of circulating gut factors leading to a suppression of islet hormone secretion. Recent reports (27, 28) have also suggested that somatostatin release from the pancreatic islets and gastric antrum may be regulated, in part, by autonomic neural signals. Previously, we observed that phentolamine, an α-adrenergic blocker, markedly reduces the effectiveness of somatostatin as an inhibitor of hormone secretion from the pancreas of dogs (6–8). Those findings, therefore, suggest that somatostatin interacts with autonomic neural signals to the islets. In view of the extensive data concerning neural control of the endocrine pancreas (25, 29, 30) and the fact that somatostatin exists within certain central and peripheral neurons (4, 31), it is additionally possible that somatostatin acts upon a center within the central nervous system that, in turn, inhibits the release of islet hormones. At present, these hypotheses seem most consistent with our experimental findings.

Summary. The inhibitory action of somatostatin upon the endocrine pancreas of dogs was studied as a function of dose and site of administration. Hormone output was measured directly in the pancreaticoduodenal vein. Infusions of somatostatin into the pancreatic artery show that a predictable dose-response relationship exists for this controller of islet function. The high relative potency of somatostatin when administered via a femoral vein or the hepatic portal vein suggests that indirect mechanisms of action are responsible, in part, for the effect of this peptide upon insulin and glucagon output from the pancreas *in vivo*. It is postulated that, in its indirect effects, somatostatin may inhibit islet hormone secretion by decreasing the circulating levels of several gastrointestinal hormones known to potentiate insulin and glucagon release, or it may elicit an inhibitory neural-pancreatic reflex.

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Tonic Sympathoinhibition in the Baroreceptor Denervated Cat[1] (40114)

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The baroreceptor reflexes have long been recognized as having a critical role in controlling the level of sympathetic nerve discharge (SND) and, thereby, maintaining blood pressure within normotensive levels (1). Sympathoinhibitory mechanisms of nonbaroreceptor origin apparently also function tonically to control SND. Alexander (2) reported an increase in SND after cervical spinal cord section in an animal in which SND had previously been maximally reduced by medullary transection at the level of the obex. The increase in SND presumably was due to release of inhibition of nonbaroreceptor origin since transection at the level of the obex is caudal to the point of entry into the medulla of the IX and X cranial nerves. Coote *et al.* (3) showed that the spinal component of the somatosympathetic reflex was augmented after section of the cervical spinal cord. It was suggested that the enhancement of the reflex was due to the release of inhibition of nonbaroreceptor origin since these investigators also reported that the reflex was not affected by baroreceptor reflex activation in cats with an intact neuraxis.

The present investigation was designed to answer three questions regarding sympathoinhibition of nonbaroreceptor origin. First, what are the sources of tonic sympathoinhibition in the baroreceptor denervated cat? Second, is tonic sympathoinhibition of nonbaroreceptor origin transmitted over a brainstem circuit distinct from that which receives baroreceptor input? Third, what are the relative contributions made by the nonbaroreceptor and baroreceptor inhibitory systems in the control of SND in the anesthetized cat?

Materials and methods. Cats weighing between 2.0 and 3.5 kg were anesthetized with an ip injection of a mixture of sodium diallylbarbiturate (60 mg/kg), urethane (240 mg/kg), and monoethylurea (240 mg/kg). Animals were paralyzed with gallamine triethiodide (4 mg/kg, iv) and artificially respired in order to prevent somatomotor movements which might accompany placement of the lesions in the brainstem. Blood pressure was monitored via a femoral arterial catheter and displayed on a Grass polygraph. Lead II of the ECG was also recorded and rectal temperature was maintained between 36 and 38°C with a heat lamp.

Baroreceptor denervation. Baroreceptor nerves were isolated in the neck as previously reported from this laboratory (4). Baroreceptor denervation was accomplished by bilateral section of the carotid sinus, aortic depressor, and vagus nerves.

Brainstem lesions and histology. Animals were placed in a David Kopf Instruments stereotaxic apparatus after which the dorsal aspect of the medulla was exposed by removal of a portion of the occipital bone and cerebellum. Medial medullary lesions were made with an electrode 0.5 mm in diameter whose tip was exposed 7 mm so that the lesion would extend from the dorsal to the ventral surface of the brainstem. Lesions were made in the midline 0–2 mm rostral to the obex in several steps by passing 5 mA of anodal current for 15 sec. The cathode was an electrode attached to the temporal muscle. Electrolytic lesions (5 mA for 15 sec) of the nucleus of the tractus solitarius (NTS) were made bilaterally 1 mm rostral and 1 mm caudal to the obex. As described by Miura and Reis (5), the posterointermediate sulcus was used as a surface landmark for localization of NTS along its rostral–caudal extent. The electrode was placed on or just medial to the sulcus and 1.3–1.5 mm below the surface of the medulla.

The extent of the electrolytic lesions of medial medullary structures and of NTS was

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ined by examination of histological
s. The medulla was removed and fixed
buffered formalin following each ex-
nt. Frontal sections of 30 μm thickness
ut with a cryostat microtome and
with cresyl violet.

rebration. Pretentorial decerebration
rformed with a spatula placed in a
ıxic electrode holder. The spatula 8
diameter was lowered first through the
de and then through the left side of
instem at the midcollicular level. The
teness of decerebration was visually
ed at the end of each experiment.

ɔathetic nerve recordings and data anal- branch of the left renal postganglionic
hetic nerve was exposed via a retro-
eal approach, as previously reported
iis laboratory (4). SND was recorded
hasically under oil with bipolar plati-
electrodes after capacity-coupled
ɔlification (low and high half-ampli-
sponses at 1 and 1000 Hz). The output
preamplifier was led to a Grass 7P10
tive integrator. Raw and integrated
were displayed on a polygraph.
ɛs in SND were quantified by compar-
mean epoch length (integrated rec-
luring 5 min periods before and at
times after medullary lesions or bar-
or denervation.

phase relations between SND and the
cycle were analyzed by computer
tion (Nicolet model 1070). The sweep
computer was triggered by a timing
ɔincident with the R wave of the ECG.
ɛd records of SND and the arterial
ʼave were displayed on an oscilloscope
ɔnix model 502) and photographed on
film.

stical analysis. Statistical analysis was
ıed with the Student *t* test for paired
paired data. *P* values of <0.05 were
ɛred to indicate statistical significance.
are expressed as mean ± SE.

lts. Medial medullary lesions. Blood
e and SND are decreased by electrical
tion of the paramedian reticular (4),
ɔbscurus and raphe pallidus (6) nuclei
nedial medulla. Whether these effects
diated over baroreceptor internuncial
ks remains controversial (5, 7–9).
ʼer, it is not known whether sympa-
bitory elements in this region are ton-
ically active in the baroreceptor denervated cat. With regard to these questions, the effects on renal SND and blood pressure produced by electrolytic lesions of medial medullary structures located 0–2 mm rostral to the obex were compared in baroreceptor intact and denervated cats.

The upper panels (A and B) in Fig. 1 show an example of the effects on renal SND and blood pressure produced by medial medullary lesions in a cat with intact baroreceptor nerve fibers. SND and blood pressure decreased (not shown) during application of anodal current. Approximately 30 min was required after the lesions were made for SND and blood pressure to stabilize. At this time (panel B), the level of renal nerve activity was considerably higher than before (panel A) placement of the lesions. As shown in Table IA, renal SND was increased to 142% of control 30 min after medial medullary lesions in four cats with intact baroreceptor nerves. Although blood pressure was somewhat elevated 30 min after placement of the lesions in the experiment illustrated in Fig. 1, the increase in SND produced by medial medullary lesions was not accompanied by a statistically significant change in arterial pressure (Table IA). No further changes in SND and blood pressure were observed for the duration of the experiment (1–2 hr).

SND normally is synchronized into bursts which are locked in a 1:1 relation to the cardiac cycle by the baroreceptor reflexes (4). The R wave-triggered computer summed records (inset) in panel A of Fig. 1 clearly depict the cardiac-related periodicity in SND. Lesions of the medial medulla which increased SND did not disrupt the phase relations between SND and the cardiac cycle (inset in panel B). Moreover, in three experiments, medial medullary lesions which led to marked increases in basal renal SND had little effect on the inhibition of SND associated with the rise in blood pressure produced by the iv injection of 1 μg/kg of norepinephrine bitartrate (Fig. 2). Gebber *et al.* (10) have previously demonstrated that norepinephrine-induced reflex inhibition of SND is eliminated by bilateral section of the baroreceptor nerves in cats anesthetized with the dial–urethane mixture used in the present study. These observations indicate that medial medullary lesions had little or no effect

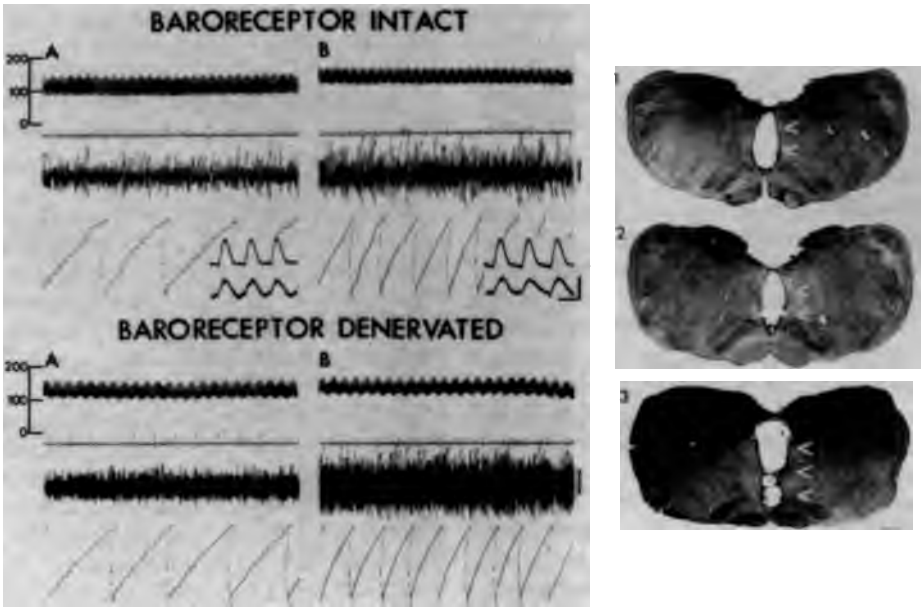


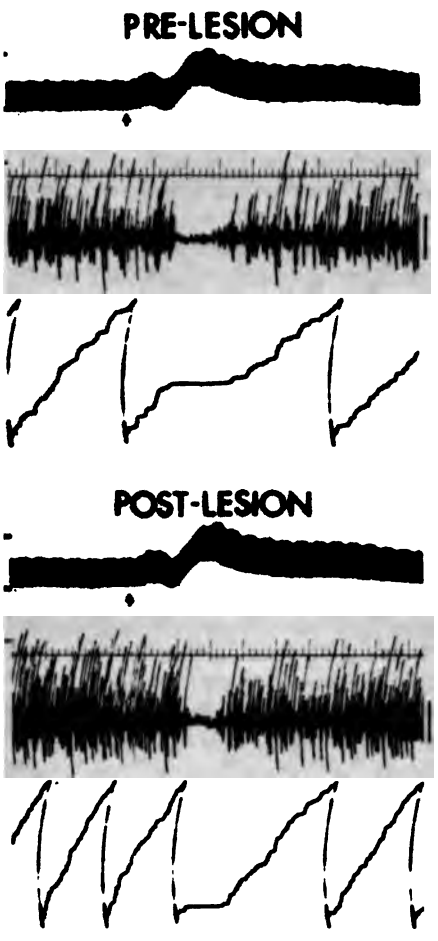
FIG. 1. Effects of medial medullary lesions on blood pressure and renal SND in a baroreceptor intact (up panels) and a baroreceptor denervated (lower panels) cat. Panels A: before lesions. Panels B: 30 min after lesic Top trace in each panel is blood pressure (mmHg). Middle trace is SND. Bottom trace shows integrated SND. Ti base (below blood pressure) is 1 sec/division. Vertical calibrations for polygraphic traces of SND are 100 μV. In in bottom traces in the experiment on baroreceptor intact cat show R wave-triggered computer summed records trials) of arterial pulse wave (top) and SND (bottom). Horizontal calibrations for computer summed records is msec. Vertical calibration is 67 μV and refers to SND. Address bin for computer summed records was 4 m Histological sections on the right show the extent of medial medullary lesions in the baroreceptor intact cat medullary section 2 mm rostral to the obex. 2: 1 mm rostral to the obex. 3: level of the obex. Bar = 1 mm. Arr point to lesions.

TABLE 1. EFFECTS OF MEDULLARY LESIONS AND BARORECEPTOR DENERVATION ON RENAL SND AND BLOOD PRESSURE.

Procedure	SND 30 min after (% of control)	Mean blood pressure Control (mmHg)	30 min after (mmHg)
A) Medial Medullary Lesions			
Baroreceptor intact	142 ± 11 (4)*[a]	123 ± 3	123 ± 7
Baroreceptor denervated[b]	161 ± 13 (11)*	133 ± 5	135 ± 4
Denervated-decerebrated	93 ± 5 (5)	137 ± 5	130 ± 4
B) Baroreceptor Denervation	140 ± 10 (10)*	138 ± 6	144 ± 7
C) NTS Lesions			
Baroreceptor intact	154 ± 13 (4)*	137 ± 8	143 ± 12
Baroreceptor denervated	109 ± 12 (5)	130 ± 11	131 ± 15

[a] Mean ± S.E. (*n*).
[b] Bilateral section of the carotid sinus, aortic depressor, and vagus nerves.
* $P < 0.05$ compared to control (paired comparisons).

on the sympathoinhibitory component of the baroreceptor reflexes. Thus, the increase in SND produced by the lesions presumably was due to the release of tonic sympathoinhibition of nonbaroreceptor origin. This contention is supported by the results obtained with medial medullary lesions in cats in which the carotid sinus, aortic depressor, vagus nerves were bilaterally sectioned. shown in Table IA, renal SND was incre to 161% of control 30 min after lesions made in 11 baroreceptor denervated This change was not significantly diffe from that produced by lesions in barorece



2. Reflex inhibition of renal SND associated
: in blood pressure produced by iv norepineph-
ug/kg) before (upper panel) and 30 min after
anel) medial medullary lesions. Sequence of
the same as in Fig. 1. Arrows indicate injection
inephrine. Time base is 1 sec/division. Vertical
on is 100 μV and refers to SND.

cats. As was the case in baroreceptor cats, the increase in SND observed 30 fter placement of the lesions was not panied by a change in blood pressure. fect on SND produced by medial med- lesions in one of the experiments per- l on baroreceptor denervated cats is in the lower panels (A and B) in Fig.

extent of the medial medullary lesions in baroreceptor intact and denervated shown by the histological sections in The lesions destroyed the caudal portions of the paramedian reticular, raphe obscurus and raphe pallidus nuclei located 0–2 mm rostral to the obex. The lesioned area did not include the medullary nucleus of baroreceptor fiber termination (i.e., NTS).

In order to determine whether sympathoinhibition involving the medial structures of the caudal medulla is dependent upon the integrity of forebrain-medullary connections, the effects on renal SND produced by lesions of the medial medulla were studied in baroreceptor denervated cats which were decerebrated at the midcollicular level. The results from five experiments are summarized in Table IA and a typical experiment is shown in Fig. 3. Medial medullary lesions failed to increase SND or blood pressure in the decerebrated cat (panel B).

Baroreceptor denervation. The foregoing data suggest that tonic sympathoinhibition involving medial structures of the caudal medulla is of nonbaroreceptor origin. It was of interest to ascertain the relative contributions made by this system and by the baroreceptor reflexes in the control of SND in the anesthetized cat. For this purpose, the effect of bilateral section of the carotid sinus, aortic depressor and vagus nerves on renal SND was studied in 10 cats. The results of these experiments were compared with those from cats in which the medial medulla was lesioned. As shown in Table IB, renal SND was increased to 140% of control 30 min after baroreceptor nerve section. This change was not significantly different from that produced by medial medullary lesions in cats with intact or with sectioned baroreceptor nerves (Table IA). Thus, the baroreceptor and nonbaroreceptor sympathoinhibitory systems were equally important in controlling the level of basal SND under the conditions of our experiments.

A typical experiment depicting the effects of baroreceptor nerve section on renal SND and blood pressure is shown in Fig. 4. Blood pressure and SND were markedly increased 5 min after bilateral section of the carotid sinus, aortic depressor, and vagus nerves (middle panel). As reported by others (1, 11–13), the hypertensive response to baroreceptor nerve section is not sustained, especially in anesthetized preparations. Although SND remained elevated for the duration of the experiment, blood pressure returned near to control level within 30 min after barore-

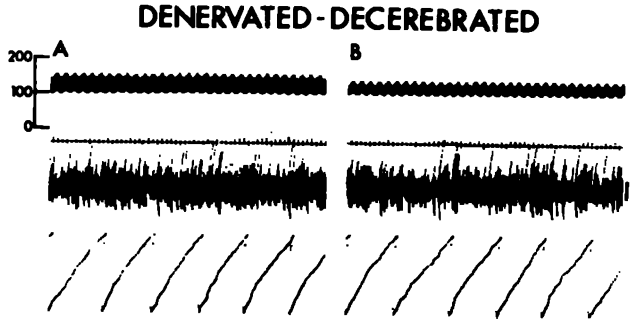


FIG. 3. Effects of medial medullary lesions on blood pressure and renal SND in baroreceptor denervated a decerebrated cat. Sequence of traces is the same as in Fig. 1. A: before lesions. B: 30 min after lesions. Time base 1 sec/division. Vertical calibration is 100 μV.

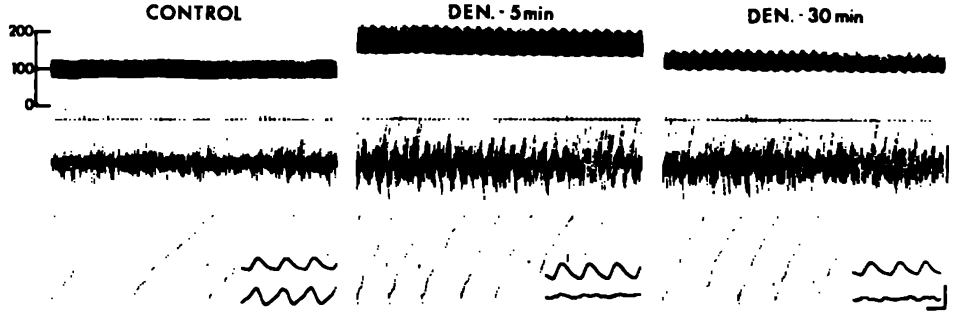


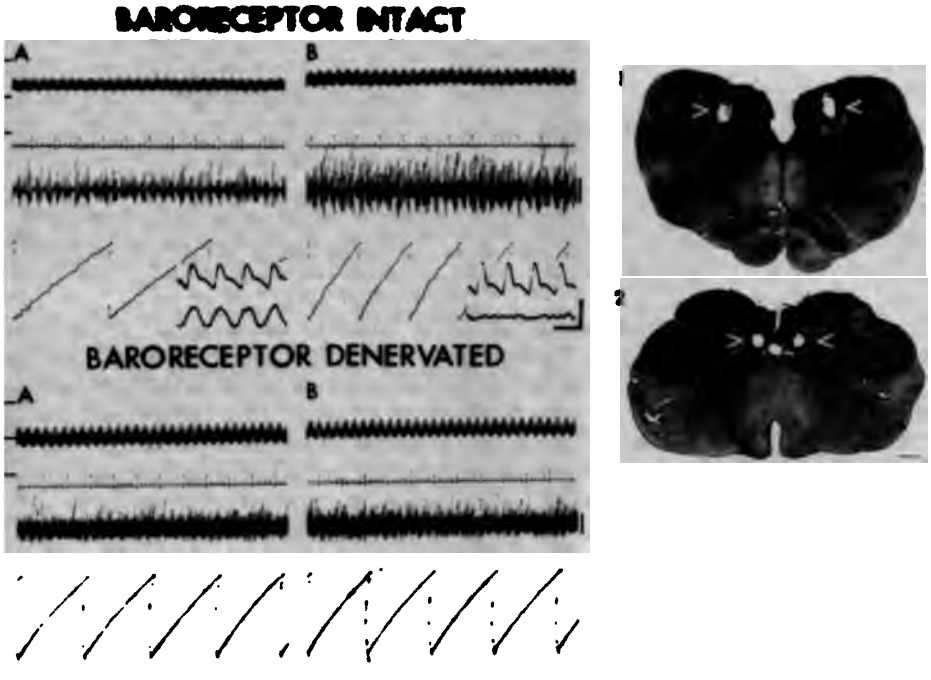
FIG. 4. Effects of baroreceptor denervation on blood pressure and renal SND. Sequence of traces is same as Fig. 1. Left: control. Middle: 5 min after bilateral section of the carotid sinus, aortic depressor, and vagus nerves Right: 30 min after baroreceptor denervation. Time base is 1 sec/division and vertical calibration is 100 μV fo polygraphic records of SND. Insets in bottom traces show R wave-triggered computer summed records (64 trials) o arterial pulse wave (top) and SND (bottom). Address bin and calibrations for computer summed records are sam as in Fig. 1.

ceptor nerve section (Fig. 4, right panel and Table IB). Baroreceptor denervation was complete in these experiments since as shown in the insets in Fig. 4, bilateral section of the carotid sinus, aortic depressor and vagus nerves disrupted the phase relations between SND and the cardiac cycle. The computer summed records of SND approached a straight line after section of these nerves since activity in the renal nerve was no longer locked in time to the R wave trigger by cardiac-related baroreceptor nerve discharge. In addition, as reported previously by Gebber *et al.* (10), section of the aforementioned nerves prevented the reflex inhibition of SND associated with the rise in blood pressure produced by the iv injection of norepinephrine (1–2 μg/kg).

NTS lesions. The intermediate one-third of NTS is the primary site of termination o baroreceptor fibers of the carotid sinus, aorti depressor, and vagus nerves in the cat (7- 14–15). Recently, it has been established tha some interneurons in NTS receive drivin inputs from nonbaroreceptor as well as fro baroreceptor afferents. Specifically, McCa *et al.* (16) found that NTS neurons in th baroreceptor reflex arc could be activated b electrical stimulation of afferents in the infe rior cardiac nerve. With regard to these find ings, it was of interest to determine whethe sympathoinhibitory interneurons in the bar oreceptor reflex arc remain tonically activ after section of the carotid sinus, aortic de pressor, and vagus nerves. For this purpos the effects of NTS lesions on renal SND wer compared in baroreceptor intact and denerv vated cats. The extent of the lesions placed i

mm caudal and 1 mm rostral to the illustrated in the histological sections 5. As shown in Table IC and in the anels (A and B) of Fig. 5, ablation of rmediate one-third of NTS signifi- ncreased renal SND in cats in which oreceptor nerves were intact. These completely disrupted the phase rela- tween SND and the cardiac cycle (see thus indicating that the sympathoin- component of the baroreceptor re- as eliminated. As was the case with medullary lesions or with section of oreceptor nerves, blood pressure was ificantly changed 30 min after NTS oned. As shown in Table IC and in er panels (A and B) of Fig. 5, NTS ailed to alter SND in the baroreceptor ted cat. This observation indicates pathoinhibitory interneurons in NTS tonically active in the absence of eptor nerve discharge.

Discussion. The present study has shown that tonic sympathoinhibition of nonbaroreceptor origin is mediated by neuronal elements which originate or pass through medial structures in the caudal medulla of the cat. These structures include portions of the paramedian reticular, raphe obscurus and raphe pallidus nuclei. Our conclusion is based on the observation that medial medullary lesions placed 0–2 mm rostral to the obex produced a significant increase in renal SND in the baroreceptor denervated cat. Lesions of this region in baroreceptor intact cats also increased renal SND without disrupting the phase relations between SND and the cardiac cycle or reflex inhibition of SND associated with the pressor action of norepinephrine. In this regard, section of the baroreceptor nerves completely disrupted the phase relations between SND and the cardiac cycle (Fig. 4) and eliminated reflex inhibition of SND produced by increasing blood pressure with norepi-



. Effects of NTS lesions on blood pressure and renal SND in a baroreceptor intact (upper panels) and a tor denervated (lower panels) cat. Sequence of traces in each panel is same as in Fig. 1. Panels A: before anels B: 30 min after lesions. Time base is 1 sec/division and vertical calibration for polygraphic traces of 00 μV. Insets in bottom traces in the experiment on the baroreceptor intact cat show R wave-triggered summed traces of arterial pulse wave (top) and SND (bottom). Address bin and calibrations for computer records are same as in Fig. 1. Histological sections on the right show extent of NTS lesions in the tor intact cat. 1: 1 mm rostral to the obex. 2: 1 mm caudal to the obex. Bar = 1 mm. Arrows point to

nephrine. Thus, it would appear that tonic sympathoinhibition from the medial medulla is transmitted through a brainstem circuit distinct from that responsible for baroreceptor reflex effects on SND.

The increase in SND produced by medial medullary lesions in baroreceptor denervated cats was prevented by prior decerebration. This observation argues against the possibility that the increase in SND produced by lesions of the medial medulla was due to irritation of surrounding sympathoexcitatory regions rather than to the release of neural inhibition. It is also clear from this result that tonic sympathoinhibition of nonbaroreceptor origin is somehow dependent upon the integrity of forebrain–medullary connections. It is possible that tonic sympathoinhibition involving midline structures of the caudal medulla is of forebrain origin. In this regard, Lofving (17) demonstrated that the decrease in blood pressure produced by electrical stimulation of the anterior hypothalamus was prevented by ablation of the medial medulla. Alternatively, the inability to produce an increase in SND with medial medullary lesions in the decerebrated cat might indicate that tonic sympathoinhibition of nonbaroreceptor origin acts specifically to suppress SND emanating from the forebrain. Concerning this possibility, it has been claimed (18) that sympathoexcitatory elements in the posterior hypothalamus are tonically active in the anesthetized cat.

The manner in which activity in the nonbaroreceptor sympathoinhibitory system is generated remains obscure. Nevertheless, it is noteworthy that the increase in renal SND produced by medial medullary lesions in cats with an intact neuraxis was not significantly different from that observed following section of the baroreceptor nerves or after lesions of the medullary nucleus of baroreceptor fiber termination (i.e., NTS). This observation suggests that the nonbaroreceptor sympathoinhibitory system is potentially as important as the baroreceptor reflexes in the control of SND in the anesthetized cat.

The increase in SND produced by medial medullary lesions was not accompanied by an increase in blood pressure. This observation undoubtedly explains why such lesions increased SND to the same extent in baroreceptor intact and denervated cats. That is, the expected increase in baroreceptor reflex activity which might have compensated for the removal of sympathoinhibition of nonbaroreceptor origin did not occur in cats with intact carotid sinus, aortic depressor, and vagus nerves since blood pressure was not changed by medial medullary lesions. At first glance it is disconcerting that blood pressure failed to rise in the face of elevated SND following medial medullary lesions. However, the observation is not so surprising in view of the results obtained with baroreceptor denervation. As noted by others (1, 11–13), the rise in blood pressure immediately following section of the baroreceptor nerves was not sustained beyond 30 min, a period equivalent to that required for SND to stabilize after placement of medullary lesions. In contrast, the increase in SND produced by baroreceptor denervation persisted for the duration of the experiment. Thus, it is apparent that in the anesthetized cat, powerful nonneural mechanisms act to compensate for changes in blood pressure associated with increased SND. It has been suggested that the rapid dissipation of the hypertensive response produced by baroreceptor nerve section might involve a decrease in cardiac output (11) and/or "whole body circulatory autoregulation" (19). These or other nonneural mechanisms might also have precluded us from viewing significant increases in blood pressure following medullary lesions which increased SND.

Finally, the present study has demonstrated that lesions of NTS increased SND only in cats in which the baroreceptor nerves were intact. This observation indicates that sympathoinhibitory elements within the medullary nucleus of baroreceptor fiber termination are not tonically active in the anesthetized cat after section of the carotid sinus, aortic depressor, and vagus nerves. Thus, auxiliary afferent input to baroreceptor interneurons in NTS (16) are insufficient to bring these cells to discharge threshold in the absence of baroreceptor nerve activity.

Summary. Lesions of those portions of the paramedian reticular, raphe obscurus and raphe pallidus nuclei which extend 0–2 mm rostral to the obex increased renal SND without affecting the baroreceptor reflexes in cats with intact carotid sinus, aortic depressor, and vagus nerves. Lesions of these medial

medullary structures in baroreceptor denervated cats produced an equivalent increase in SND. The effect of medial medullary lesions on SND, however, was prevented by prior decerebration. These results indicate that tonic sympathoinhibition involving the medial medulla is of nonbaroreceptor origin and is dependent upon the integrity of forebrain–medullary connections. The increase in SND produced by medial medullary lesions was not significantly different from that produced by section of the baroreceptor nerves or by ablation of the medullary nucleus of baroreceptor fiber termination (i.e., NTS). Thus, the nonbaroreceptor sympathoinhibitory system of the medial medulla is potentially as important as the baroreceptor reflexes in the control of SND in the anesthetized cat. Finally, this study also demonstrated that sympathoinhibitory elements in NTS are not tonically active in the absence of baroreceptor nerve input.

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The Effect of Bran on Dimethylhydrazine-Induced Colon Carcinogenesis in the F (40115)

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The putative role of dietary fiber in preventing colon cancer has generated a great deal of interest and controversy (1, 2). Epidemiological studies indicate that large bowel diseases, particularly colon cancer, have a characteristic geographic distribution. Low incidences of colon cancer are found where the population consumes a diet high in fiber derived from natural, unprocessed foods. On the other hand, the highest incidences occur in areas where people consume highly refined diets low in fiber and high in fats.

There has been a paucity of animal studies designed to test whether or not dietary fibers indeed have a protective role in chemically-induced colon carcinogenesis. Dimethylhydrazine (DMH), a potent carcinogen with an organotropism for the colonic mucosa of the rat, provides a reliable animal model for such studies (3, 4). The present preliminary study utilized this model to determine whether incorporating wheat bran, a model dietary fiber, into the diet of rats influenced the production of colonic tumors.

Materials and methods. Male Sprague–Dawley albino rats obtained from Charles River Breeding Laboratories, Inc. (approx. 200 g body weight) were used. All animals were housed two or three together in plastic boxes lined with clean hardwood chip bedding (Betta Chip), changed twice weekly, and were allowed free access to food (ground Wayne Lab-Blox or 20% bran diet) and tap water. Ten rats each were randomly assigned to one of four groups: Group I—Control; Group II—30 mg/kg DMH; Group IlI—20% Bran; and Group IV—Bran and DMH.

The bran diet was prepared in 20 kg batches by mixing ordinary wheat bran (Agway, Inc.) into the standard diet at a concentration of 20%. Although bran diluted the components of the standard diet, note must be made that wheat bran contains 14% protein, 3% fat, and numerous vitamins and minerals as well as 11% *crude* fiber. Accord Southgate (5), bran contains 42% *diet* ber, itself composed of 7.6% cellulosic saccharides, 2.9% lignin, and 31% no losic polysaccharides. The *crude* fiber c of the standard diet was 3.2%, whereas tion of 20% bran increased the *crud* content to 4.5%.

Symmetrical 1,2-dimethylhydrazine drochloride (DMH, 97% pure) obtaine Aldrich Chemical Co. was dissolved i NaCl solution (15 mg/ml total salt given weekly by stomach tube at 30 r body weight for a total of 10 doses duri first 9 weeks of the study. The oral ro administration was chosen in this stu parallel more closely the exposure of n possible dietary carcinogens. The tw maining groups not receiving DMH similarly given 0.9% NaCl solution alo

Individual body weights were rec weekly, and daily food consumption w culated for each rat thrice weekly. Int transit times and fecal output were esti during the 16th, 17th and 20th weeks study. For this purpose, rats were indivi housed in metabolism cages for a 1 acclimation period, and were allowe access to food and water. A 2% suspens carmine dye in 0.9% NaCl solution wa given orally by stomach tube (5 ml/kg weight) to each rat in Groups I and serve as a fecal marker. Beginning 11 h intubation of the dye, the feces were ob hourly, and the first appearance of t dye in the fecal pellets was recorded. output was estimated in all groups as th wet weight of feces collected in a 24-hr from each rat.

The rats were killed at 21 weeks overdose of ether, 12 weeks after the la of DMH. The intestinal tract was o longitudinally and carefully examin polypoid or sessile tumors. Raised ar

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olonic mucosa, especially near Peyer's es, which were not obvious as neo- is grossly were all sampled for micro- confirmation. In addition, a represent- number of the colonic and duodenal rs were fixed in cold 70% ethanol-10% red formalin–glacial acetic acid (20:2:1) 0% neutral buffered formalin, and dded in paraffin. Sections 4 μm thick stained with H&E and PAS for histo- logical examination.

tistics. Data was expressed as mean ± ll statistics were done using Student's *t*

ults. Gross observations. Rats from all s were generally normal in appearance ehavior throughout the 21-week study. ia was seen around the anus of a few r-bearing animals, and one rat from p IV was found dead at 3 weeks, appar- due to accidental intubation of the [solution into the lungs.

dy weights and food consumption. The body weights (Table I) of rats in ps II and IV were lower ($P < 0.05$) than ol (Group I) at 5 weeks. This trend was pronounced at 10 weeks ($P <$ 0.001), and may reflect the toxicity of [. Following cessation of DMH treat- body weight gains in both groups given [equalled or exceeded that of the Con- group. The reason for the depressed h rate in rats given the 20% bran diet is nown at this time, but may have been o adsorption to the fiber of trace ele- essential for growth (6). Food con- tion varied from day to day within and en groups, but overall average daily consumption of the standard diet was 28 compared to 34 g/rat for the 20% bran

Intestinal transit time and fecal output. The transit time-50% for rats given the standard diet was 18 hr compared to ≤11 hr for rats given the 20% bran diet. Mean transit time was also decreased ($P < 0.05$) in the same group. Rats in both groups on the 20% bran diet excreted more feces ($P < 0.05$) than those fed the standard diet (Table II). The average increase in fecal output was greater than the amount of bran in the diet, and so was not simply the result of indigestable fiber in the 20% bran diet.

Tumor incidence and distribution. (Table III.) Gastrointestinal tumors were not found in rats belonging to Groups I and III. Rats given DMH alone had a 100% (10/10) incidence of grossly observable colonic tumors with a mean of 6.4 tumors/rat and a range of 3–12 per rat. There was a slight decrease in the incidence of colonic tumors in rats fed 20% bran and given DMH (67%, 6/9), and a lower ($P < 0.001$) mean of 1.8 tumors/rat/group with a range of 0–5 per rat (Table III). Also, the number of colonic tumors found in rats that had tumors in this

TABLE II. INTESTINAL TRANSIT TIMES AND MEAN FECAL OUTPUT.

Group	Transit time-50%[a] (hr)	Mean transit time (hr)	Fecal output[c] (g)
Control	18	18.6 ± 1.43[b]	6.0 ± 0.68
DMH			7.7 ± 1.06
Bran	≤11	13.9 ± 1.16*	10.0 ± 1.33*
Bran & DMH			9.6 ± 1.09*

[a] First appearance of red-colored fecal pellets in 50% of the rats in each group.

[b] Transit time in one rat was >24 hr; arbitrarily estimated to be 25 hr.

[c] Wet weight/24 hr.

* Statistically different ($P < 0.05$) from Control group.

TABLE I. MEAN BODY WEIGHTS (g) OF RATS GIVEN BRAN, DMH, OR BRAN AND DMH.

Group[a]	Weeks on Test					
	0	2	5	10	15	20
ıtrol	192 ± 4	297 ± 4	406 ± 6	499 ± 6	567 ± 9	585 ± 15
[H	192 ± 3	291 ± 5	378 ± 10*	451 ± 13**	534 ± 15	581 ± 16
n	201 ± 4	293 ± 5	392 ± 9	474 ± 12	530 ± 20	563 ± 26
n & DMH	201 ± 3	289 ± 6	372 ± 14*	440 ± 13***	507 ± 12**	527 ± 21*

tistical differences from Control group.
< 0.05.
< 0.01.
< 0.001.

TABLE III. EFFECTS OF BRAN ON DIMETHYLHYDRAZINE-INDUCED INTESTINAL TUMORS IN RATS.

	Colonic tumors		Duodenal tumors		Cecal tumors	
Group	Incidence	No./Rat[a]	Incidence	No./Rat[a]	Incidence	No./Rat[a]
DMH	10/10 (100%)	6.4 ± 0.88 (3–12)	8/10 (80%)	1.3 ± 0.33 (0–3)	1/10 (10%)	0.2 ± 0.20 (0–2)
Bran and DMH	6/9 (67%)	1.8 ± 0.62*[b] (0–5)	8/9 (89%)	2.2 ± 0.55 (0–5)	2/9 (22%)	0.2 ± 0.15 (0–1)

[a] Mean ± SE for group (range).
[b] Mean number of tumors/rat with tumors was 2.7 ± 0.67 ($P < 0.01$).
* Statistically different ($P < 0.001$) from DMH group.

group was 2.7 tumors/rat, still lower ($P < 0.01$) than that found in Group II. However, the size (mean 3.7 mm diam, range 1–10 mm) and distribution (mean 10.7 cm proximal to anorectal junction) of the colonic tumors were similar between Groups II and IV.

The incidence of duodenal tumors was similar between the two dietary groups. The number of duodenal tumors in Group IV was slightly higher (2.2 tumors/rat/group), but not significantly different from Group II (1.3 tumors/rat/group). The size (mean 5.7 mm diam, range 1–14 mm) and distribution (mean 2.4 cm distal to pyloric junction) of these tumors were similar in both groups.

In addition, two tumors were found in the cecum of one rat from Group II, and one cecal tumor was found in two rats from Group IV.

Histopathology. In general, random and representative tumors from Groups II and IV were subjected to histopathologic examination. Polypoid adenomas, adenocarcinomas, and mucinous adenocarcinomas were noted in agreement with earlier reports (7). No attempt was made to characterize histologically every tumor counted grossly. One interesting feature, though, was a striking absence of PAS-positive material in the polypoid adenomas which correlated with the relative absence of mature goblet cells.

Discussion. There is a group of diseases characteristic of Western civilization which has been linked to fiber-depleted diets. These diseases include diverticular disease of the colon, appendicitis, hiatus hernia, hemorrhoids, varicose veins, and cancer of the colon and rectum (1). Burkitt suggests that the occurrance of bowel cancer and fiber depletion is a "reasonably well-supported hypothesis", but little experimental data in animals is available to support or reject this hypothesis. Dietary fibers are known to affect the bacterial flora of the gut (8), and effects on intestinal transit time and fecal output are well documented (9).

In the present experiment, and in keeping with previous reports in man, dietary bran decreased intestinal transit time and significantly increased fecal output. The time allowable for bacterial activation of postulated cocarcinogens would therefore be reduced, and the greater fecal bulk would tend to dilute the carcinogen, as well as bile salts, the latter recently implicated as cocarcinogens (10, 11). These effects were associated with a reduction in the incidence and number of colonic tumors in rats, suggesting that this dietary fiber provides partial protection against chemically-induced colon carcinogenesis.

The number and incidence of duodenal tumors were similar between dietary groups. The close proximity of these tumors to the opening of the common bile duct suggested that an active metabolite of DMH was being excreted directly into the bile, or that the high local concentration of bile salts was acting to promote tumorigenesis in this area. It should be mentioned that the mechanism of carcinogenesis of DMH has a significant systemic component (12). The active metabolite of DMH, methylazoxymethanol, can reach the epithelial cells of susceptible mucosa via the systemic circulation independent of the lumenal contents of the gut. The fact that bran probably exerts its major physiological effects in the colon may explain the absence of any reduction in the mean number of duodenal tumors in rats given the bran diet.

The results of this study are in agreement with Wilson *et al.* (13) who noted that bran reduced the incidence of colon tumors induced by DMH in rats fed diets high in beef fat or corn oil. On the other hand, Ward *et*

.4) found that while alphacel (refined lose) had little effect on the number of ı tumors, small intestinal tumors were ɔed by addition of high levels of this to the diet. The differences in the mode lministration of DMH and the type of used are probably the major reasons for liscrepancy. Cellulose is only one chem-ˈ-defined component of dietary fiber, ː bran is a substance with complex nu-nal and chemical characteristics (15). modest increase in *crude fiber* of the 20% diet should not obscure the fact that the *ry fiber* content is qualitatively different much greater scale.

mmary. The putative role of dietary fiber investigated in dimethylhydrazine H)-induced colon carcinogenesis in rats. groups of 10 male Sprague–Dawley rats were used: Group I—Control; Group 0 mg/kg DMH; Group III—20% bran; Group IV—Bran and DMH. Intestinal it times were decreased and fecal outputs increased in rats given the 20% bran In Group II, all 10 rats had tumors with an of 6.4 tumors/rat whereas in Group of 9 rats (67%) had tumors with a mean ıly 1.8 tumors/rat/group and 2.7 tu-/rat which had tumors. The size and bution of these tumors were similar in groups. Bran in the diet had little effect e incidence, number, and distribution of enal tumors.

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Sparsomycin-Induced Disaggregation but not Detachment of Hepatic Membrane-Bound Polyribosomes[1] (40116)

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For a number of years our laboratory has been concerned with the mechanisms by which selected hepatotoxic agents act on hepatic polyribosomes and protein synthesis in experimental animals. Among the agents investigated were actinomycin D (1–3), puromycin (4–6), ethionine (5, 7–9), aflatoxin B_1 (10), CCl_4 (5, 8, 11), hypertonic NaCl (12–15), dimethylnitrosamine (16), and sparsomycin (5, 17). The studies were concerned with how these agents affect the total polyribosomes as well as the free and membrane-bound polyribosomes of the livers of rats and mice. Studies dealing with the nature of attachment of ribosomes to membranes in livers of animals exposed to hepatotoxins were also undertaken. They revealed that, even though agents such as ethionine, puromycin, CCl_4, and sparsomycin caused extensive disaggregation of membrane-bound polyribosomes, they did not cause detachment of ribosomes from the membranes (5). This led to further studies which revealed that normal membrane-bound ribosomes attached to membranes could be influenced *in vitro* by $[K^+]$ (increasing $[K^+]$ leads to detachment) (8). Similar findings have been reported by Adelman *et al.* (18) who also demonstrated that ribosomes are bound to membranes by a direct interaction between the membrane and the large ribosomal subunit which are labile at high KCl concentrations. Also, evidence has been presented that disaggregated membrane-bound ribosomes obtained after *in vivo* treatment of mice or rats with ethionine or CCl_4 could be released from membranes with high KCl concentrations (8).

Recently we investigated the effect of $[K^+]$ *in vitro* on detachment of hepatic ribosomes after sparsomycin treatment *in vivo* and observed that the ribosomal attachment to membranes was different than that found earlier after ethionine or CCl_4. Sparsomycin has been demonstrated *in vivo* to cause marked disaggregation of hepatic polyribosomes (5, 17, 19, 20). The present report deals with the findings which suggest that the hepatic ribosomes on membranes after sparsomycin treatment *in vivo* are attached via peptides as well as by ionic bridging influenced by $[K^+]$.

Materials and methods. Female mice weighing 25 g and female rats weighing 150–180 g (Hilltop Lab Animals, Inc., Scottdale, PA) were fasted overnight before using.

Labeled membrane-bound polyribosomes were obtained by administering [^{14}C]-6-orotic acid (58 mCi per mmole), 2.5 μCi per mouse or 5 μCi per rat, intraperitoneally 20 hr before killing.

Sparsomycin, obtained as a gift from the National Cancer Institute, 1 μg/g of body weight, was administered intraperitoneally 2 hr before killing. Dimethylnitrosamine, obtained from Eastman Organic Chemicals, 1.25 mg/mouse was administered intraperitoneally 1 hr before killing. Control animals received saline (0.9% NaCl).

Preparation of membrane-bound ribosomes and determinations of radioactivity in the ribosomes were essentially the same as described earlier (5, 8). Ten milliliter aliquots of 1.38 *M* sucrose layer (containing 0.05 *M* Tris, pH 7.6, and 0.005 *M* $MgCl_2 \cdot 6H_2O$ (TM), rat liver cell sap, and membrane-bound polyribosomes freed of free ribosomes) were adjusted to different concentrations of KCl (2.5 *M* KCl in 0.25 *M* sucrose containing

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TM was added to give final concentrations of 0.1, 0.2, or 0.4 *M* KCl). Total volumes were made up to 12.5 ml with TM and the tubes were incubated on ice for 20 min and 12.5 ml of incubating mixture were loaded on to a discontinuous gradient of 7 ml of 2.0 *M* sucrose in TM and 7 ml of 1.38 *M* sucrose in TM. The tubes containing 26.5 ml were centrifuged for 20 hr at 29,000 rpm at 2° in a 30 rotor in a Spinco model L3-40 centrifuge. The 1.38 *M* sucrose layer and above supernatant was aspirated, treated with 10% deoxycholate to give a final concentration of 1% deoxycholate, diluted with TKM (TM + 0.025 *M* KCl) to 30 ml and centrifuged for 4 hr at 40,000 rpm at 2° in a 42.1 rotor to pellet membrane-bound ribosomes. The 2.0 *M* sucrose layer along with the pelleted detached ribosomes was diluted three times its volume with TKM and centrifuged 2 hr at 40,000 rpm at 2° in a 42.1 rotor to pellet detached ribosomes. One aliquot (10 ml) of 1.38 *M* sucrose layer (containing TM, rat liver cell sap, and membrane-bound polyribosomes) was incubated with puromycin (6 mg per tube), 4 mg ATP, 2.25 mg GTP, 26 mg PEP, 0.015 ml pyruvate kinase (60 I.U.) and 2.5 ml dialyzed liver cell sap for 30 min at 37° before it was incubated on ice with different concentrations of KCl. In each case, the radioactive detached ribosomes (2 *M* sucrose and pellet) and retained or undetached ribosomes (1.38 *M* sucrose layer) were determined. Percentage of detachment was calculated as detached ribosomes × 100/total ribosomes (detached + undetached). Each value was corrected for blanks.

Incorporation of [^{14}C]phenylalanine into polypeptide using ribosomes obtained from postmitochondrial supernatants under the direction of poly(U) was performed as described earlier (21).

Results. Previously we reported that membrane-bound polyribosomes of normal fasted animals prepared by the method of Blobel and Potter (22) and incubated in the presence of 0.4 *M* KCl *in vitro* revealed a 30% detachment of ribosomes while membrane-bound polyribosomes of CCl_4- or ethionine-treated animals prepared and incubated similarly revealed a 53–65% detachment of ribosomes (8). Thus, even though the membrane-bound hepatic ribosomes had become disaggregated by CCl_4 or ethionine treatment *in vivo*, they were still attached to membranes and only after KCl incubation *in vitro* became detached (8). In contrast to these findings, when rats were treated *in vivo* with sparsomycin, which caused marked disaggregation of hepatic polyribosomes (5), incubation of membrane-bound ribosomes with KCl *in vitro* failed to increase the detachment of ribosomes over that in controls (Table I). This finding led us to explore why the disaggregated polyribosomes of the sparsomycin-treated rats behaved differently than did those of CCl_4- or ethionine-treated rats.

Earlier findings suggested that membrane-bound ribosomes were attached to membranes in at least two ways: when engaged in protein synthesis, they are attached to membranes by growing polypeptides as well as by binding that can be influenced by [K^+]; and when they are not engaged in protein synthesis, they are attached only by the latter (8, 18). This suggested to us that conceivably the disaggregated ribosomes of sparsomycin-treated animals may still be attached via peptides even though they are not active in protein synthesis. To test for this we conducted experiments whereby hepatic membrane-bound ribosomes of control and sparsomycin-treated mice were preincubated *in vitro* with puromycin before incubating them with increasing concentrations of KCl. Puromycin reacts with ribosomal bound peptidyl-tRNA

TABLE I. INFLUENCE OF DIFFERENT CONCENTRATIONS OF KCl ON THE DETACHMENT OF RIBOSOMES FROM MEMBRANE-BOUND POLYRIBOSOMES OF LIVERS OF CONTROL (SALINE) AND SPARSOMYCIN-TREATED RATS.[a]

KCl *M*	Percentage of detachment of ribosomes	
	Control	Sparsomycin
0.10	13.5 ± 1.9	13.0 ± 1.9
0.20	15.7 ± 3.0	19.0 ± 2.2
0.40	22.5 ± 3.2	27.0 ± 2.7

[a] Rats received intraperitoneally as follows: control, saline 4 hr before killing or sparsomycin (1 μ/g of body weight) 2 hr before killing. Each rat received 5 μCi of [^{14}C]-6-orotate intraperitoneally 18–20 hr before killing. Membrane-bound polyribosomes (1.38 *M* sucrose layer containing 0.05 *M* Tris, pH 7.6, and 0.005 *M* Mg^{2+} and rat liver sap) were prepared and incubated with different concentrations of KCl and detached ribosomes were obtained. Groups of each experiment consisted of two rats. The results are the average of seven experiments ± SE of the mean.

TABLE II. INFLUENCE OF PUROMYCIN AND DIFFERENT CONCENTRATIONS OF KCl ON THE DETACHMENT OF RIBOSOMES FROM MEMBRANE-BOUND POLYRIBOSOMES OF LIVERS OF CONTROL (SALINE), SPARSOMYCIN-, OR DIMETHYLNITROSAMINE-TREATED MICE.[a]

Group	No. of experiments	Puromycin incubation	Percentage of detachment of ribosomes		
			0.1 *M* KCl	0.2 *M* KCl	0.4 *M* KCl
Control	3	−	9.9 ± 1.7	15.4 ± 2.0	22.8 ± 3.2
		+	13.6 ± 2.6	22.3 ± 2.1	45.3 ± 4.3
Sparsomycin	3	−	13.4 ± 1.5	19.9 ± 2.1	24.6 ± 5.2
		+	15.1 ± 1.1	29.4 ± 3.6	47.5 ± 3.6
Control	2	−	5.8 ± 3.4	10.0 ± 3.0	15.4 ± 5.6
		+	14.8 ± 6.1	30.6 ± 2.2	47.6 ± 12.4
Dimethylnitrosamine	2	−	9.3 ± 0.6	18.0 ± 3.9	23.0 ± 5.1
		+	16.4 ± 1.5	33.6 ± 6.7	56.5 ± 4.1

[a] Aliquots (10 ml) of 1.38 *M* sucrose layer (containing TM, liver cell sap, and membrane-bound polyribosomes) were incubated with 6 mg puromycin, 4 mg ATP, 2.25 mg GTP, 26 mg PEP, 0.015 ml pyruvate kinase and 2.5 ml dialyzed liver cell sap for 30 min at 37° before they were incubated on ice with different concentrations of KCl. The results are the means ± SE of the means.

to form a peptidyl puromycin product which is then released from the polyribosome (23). The results, summarized in Table II, revealed that puromycin preincubation accentuated the release of ribosomes from the membranes of the sparsomycin-treated group. This suggests that the disaggregated ribosomes of the experimental animals contain peptides which are maintaining the attachment of ribosomes to the membranes.

In one experiment we studied the ability of isolated ribosomes from sparsomycin-treated rats and control rats with poly(U) to incorporate [^{14}C]phenylalanine into polyphenylalanine. The ribosomes of the experimental group incorporated 55% less than that of controls.

Discussion. Our present findings that the sparsomycin-induced disaggregation of polyribosomes into monomers that still retain peptides which may become liberated by puromycin treatment *in vitro* are consistent with results reported by Hayes *et al.* (20). These investigators presented evidence that there was an increase in the tRNA content of hepatic total ribosomes of sparsomycin-treated mice in comparison with that of run-off ribosomes. Thus, they considered the disaggregated polyribosomes due to sparsomycin to be fall-off ribosomes (ribosomes falling off from mRNA). Also, by measuring dissociability of these 80 S ribosomes in 0.3 *M* KCl, we (8), as well as they (20), found that they dissociated into two ribosomal subunits, suggesting that the ribosomes were free of mRNA.

Our present results emphasize that certain hepatotoxic agents may *in vivo* disaggregate membrane-bound polyribosomes into monomers which structurally may be different from those induced by other agents. For example, administration of hepatotoxins such as ethionine and CCl_4 lead to disaggregation of polyribosomes to monomers that do not contain peptides (run-off ribosomes) (5). On the other hand, administration of an agent such as sparsomycin leads to disaggregation of polyribosomes of monomers that still contain peptides (fall-off ribosomes) (20). Although both classes of ribosomes under these experimental conditions do not maintain protein synthesis, the attachment of the single ribosomes of the two types to membranes are different: the former type maintains attachment by a cationic type of bridging while the latter type has, in addition, peptides which influence attachment.

Sparsomycin, a sulfur-containing antibiotic, inhibits protein synthesis in both bacterial and mammalian cells (24). It has been found to block peptide bond formation and thus prevents the transfer of amino acids from tRNA to the nascent polypeptide chains based upon previous studies using a cell-free system from *Escherichia coli* (25) and from mouse liver (19). These studies suggested that sparsomycin binds to the larger ribosomal subunits and inhibits peptidyl transferase, an enzyme responsible for peptide bond formation. Also, sparsomycin has been reported to inhibit *in vivo* the factor-dependent initiation of new polyphenylalanine chains as well as factor-dependent binding of either phe-tRNA or met-tRNA to 40 S ribosomal sub-

units (17). Using cycloheximide, an agent that acts to interfere with peptide chain release (26) and has been used to determine whether it can cause a reformation of polyribosomes previously disaggregated by hepatotoxic agents (27), we observed that it could not reassemble the ribosomes disaggregated by sparsomycin (17). Our present findings suggest that sparsomycin causes fall-off ribosomes which are defective as indicated by the decreased formation of polyphenylalanine when assayed *in vitro* with poly(U). Thus, sparsomycin appears to act in a number of ways in disturbing the protein synthetic mechanisms in the liver.

Since Chiga *et al.* (20, 28) reported that dimethylnitrosamine, as well as sparsomycin, acts *in vivo* to induce fall-off ribosomes, it became of interest to test how dimethylnitrosamine would act in relation to ribosomal attachment to membranes. Our results (Table II) reveal that it was necessary to use puromycin as well as KCl to cause detachment of ribosomes *in vitro* from membranes, results identical to those found with sparsomycin (Table II). Thus, at least two hepatotoxic agents seem to behave in a similar manner in their effect on ribosomes.

There appears to be at least three ways by which polyribosomes may breakdown into ribosomes depending upon the inducing agent. They are as follows: (a) run-off ribosomes, which occur normally or after treatment with agents such as ethionine and CCl_4 (5, 29, 30); (b) mRNA containing ribosomes, 80 S ribosomes still retaining mRNA, which are found after RNase digestion (31); and 3) fall-off ribosomes, which are found after treatment with sparsomycin or dimethylnitrosamine. Our current study presents support for the existence of this third category of ribosomes.

Summary. The nature of attachment of hepatic ribosomes to membranes after sparsomycin treatment *in vivo* of rats and mice was investigated. The results based upon the influence of treatment *in vitro* with different concentrations of KCl and with puromycin on the detachment of membrane-bound ribosomes from membranes of livers of sparsomycin-treated animals revealed that these ribosomes are attached to membranes by peptides as well as by ionic bridging and are considered as fall-off ribosomes. Similar effects have been described after treatment *in vivo* with dimethylnitrosamine. In contrast, treatment *in vivo* with ethionine or CCl_4 produced run-off ribosomes which are attached only by ionic bridging since they become detached *in vitro* with KCl treatment alone.

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Renin and Aldosterone Levels in Dolphins and Sea Lions (40117)[1]

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ine cetaceans are unique in the mam- world in that most species are never d to fresh water. However, little atten- as been directed at ascertaining the logical control mechanisms in the reg- ı of water balance in these animals. gh some animals may drink small vol- ɔf sea water, their predominant source er is their food. A similar situation egarding salt intake. Thus there is little : to vary the ratio of salt to water in iet. What then, are the control mech- responsible for maintaining the salt ater balance in these marine animals? as been shown that marine cetaceans neasurable circulating levels of renin l urinary aldosterone (2), although no strable levels of ADH have been found ıe presence of both renin and aldoster- consistent with the hypothesis that ıormones are involved in the regulation and water balance. To date, there has ıo information regarding variations in /els of either of these hormones with :e of excretion of Na or, indeed, with here is also no evidence that the renin- ensin system stimulates aldosterone se- ı in marine cetaceans and pinnipeds. ıe renin–angiotensin system is a stimu- ldosterone in these animals, as it is in ɔther mammals, one would expect the ɔrmones to vary together as the diet of imals is altered. In addition, if these ɔnes play some role in the regulation of cretion, one would expect a negative n between the rate of sodium excretion, nary concentration of Na, and the ı levels of both renin and aldosterone Accordingly, the following experiments lone to determine if such a relation in these animals.

hods. The dolphins were adult female *ɔs* sp. maintained in salt water pools at Sea World in San Diego, California since their collection in the Gulf of California near San Felipe, Mexico in the mid 1960's. The dolphins were all sexually mature, around 3 mm in length and maintaining weights of 300 ± 50 kg during the year prior to the study. Two different types of experiments were done. First, the animals were fed each morning at the same time. On day one the animals were fed their normal fish diet and a blood sample was collected 3 hr later. To do this the pool was drained, the animals were placed on a foam rubber pad and kept moist while the sample was collected from the tail fluke (2). Blood sampling required only a few minutes after which the water was raised to its normal level. Salt was fed to the animals in gelatin capsules that were placed in their food fish. Blood samples were collected at the same time each day, before, during and after the salt feeding days and fasting days.

In the second type of experiment two of the dolphins were taken from the pool and laid on a foam rubber mattress. A urinary catheter with inflatable cuff was placed in the urethra for collection of all urine for analysis and volume measurements. Blood samples were taken at the start and hourly for 8 h. Urine volume was measured hourly and an aliquot kept for analysis. Four to six liters of fresh water were given by stomach tube about one hour after the first blood sample was taken. The experiment was done in April and again in October employing the same two animals.

All blood was collected in heparinized syringes and immediately centrifuged. The plasma was separated and frozen for analysis. The sea lions were 3–6 years old males, none of which had reached sexual maturity. All animals had been acquired in a sick condition from southern California beaches and rehabilitated at least 1 year prior to the experiment. Two of the sea lions were maintained in fresh water pools and two in salt water pools at the Marine Life Sciences Laboratory

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of the Naval Undersea Center in San Diego. No differences in hormone levels were noted between the two groups.

Plasma renin activity (PRA) and concentrations of aldosterone were estimated using radioimmunoassay methods. PRA was determined by a modification of the method of Haber *et al.* (6). Samples were incubated for 20 and 40 min to insure linear generation of angiotensin I. Aldosterone was measured using the New England Nuclear Radioimmunoassay Pak (New England Nuclear, Worcester, MA). All samples were done in quadruplicate. Na was determined by flame photometry. Creatinine was measured by the method of Bonsnes and Taussky (8).

Results. Figure 1 shows the results of a single experiment in which plasma from a female dolphin (*Tursiops* sp.) was incubated in the absence and in the presence of additional dog substrate, or with the addition of hog renin. These data show that generation of angiotensin was linear with time, up to approximately 60 min and then fell off. The addition of substrate to the plasma had no effect on the generation rate of angiotensin, indicating, that in the experiments reported here, substrate was not limiting, the PRA values are a good measure of the renin concentration in the samples. If hog renin was added to the plasma, the generation rate of angiotensin was also linear for 60 min, but each point was considerably above that obtained without the addition of hog renin.

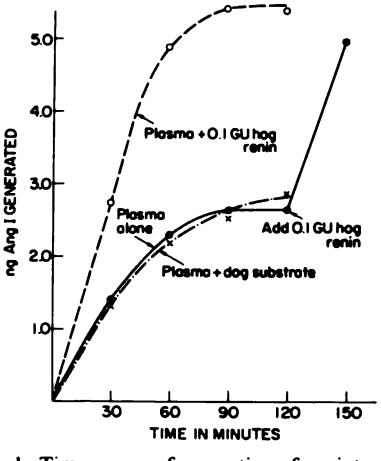


FIG. 1. Time course of generation of angiotensin I in cetacean plasma in the presence and absence of exogenous hog renin or dog substrate.

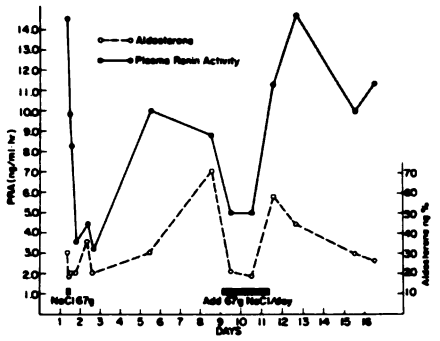


FIG. 2. Variations in PRA and aldosterone concentrations in a dolphin over a 15-day period. Horizontal bars indicate time when the animal was fed. Intervals between bars represent the fasting period.

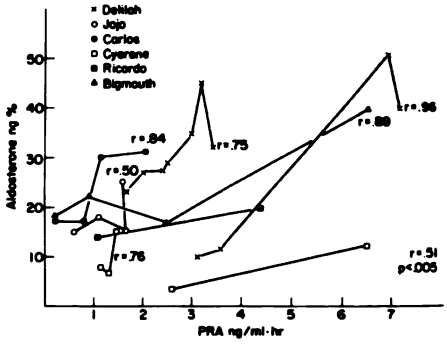


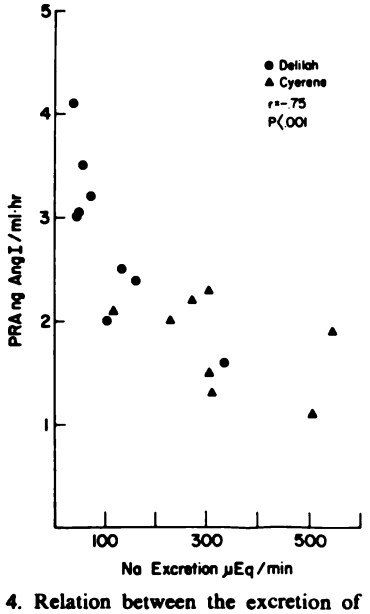
FIG. 3. Relation between PRA and aldosterone levels in six marine mammals. Carlos, Jojo, Ricardo and Bigmouth are sea lions and Delilah and Cyerene are dolphins. Lines connect data points obtained from each animal in one experimental series lasting 16 days. PRA and aldosterone values were varied by feeding NaCl (0.25 g/kg) on days 9–12. In two animals the experiment was repeated 7 months later. In these animals the two sets of data points are represented by two separate lines using the same symbols.

In Fig. 2 are plotted the results for a single animal in which plasma was sampled periodically over a 15-day period, during which the animal was subjected to periodic fasts and periodic treatment with sodium chloride. As can be seen from this figure, the alterations in the levels of aldosterone and PRA follow a similar course. It is clear that both levels fell and rose together over the 15-day period. This animal was treated differently from the animals presented in Fig. 3 in which are plotted the values obtained from all the other animals in which PRA and aldosterone were measured. For most animals there was a significant correlation coefficient between PRA

the concentration of aldosterone of ma. In addition, the overall correlation ficient was 0.51 with a P value less than 5.

igure 4 shows the results of two experi- its on dolphins in which blood and urine e sampled over a 7-hr period while the retion of sodium in the urine and PRA e also measured. There was an excellent ative correlation between the rate of Na retion and PRA. No such correlation was ained when PRA in these same animals plotted against the simultaneous endog- us creatinine clearance (Fig. 5).

Discussion. The data presented here sup- t the hypothesis that plasma renin activity aldosterone play a role in the regulation he salt balance in marine mammals. The hly significant correlation between plasma in activity and the aldosterone levels of h animal supports the hypothesis that an- ensin is a stimulator of aldosterone secre- in these species. Furthermore, it appears n these data that renin secretion may be ulated by some function of excreted Na, t is, when PRA levels are high, the rate of ium excretion is low, and vice versa. How- r, no significant relation was found be- en creatinine clearance and PRA. Thus it ifficult to assess the nature of the stimulus



IG. 4. Relation between the excretion of Na and in two dolphins.

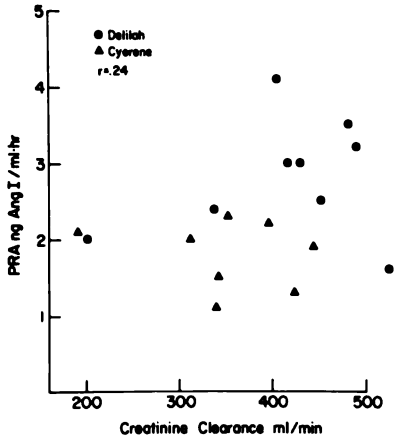


FIG. 5. The lack of correlation between the clearance of creatinine and PRA. Data are from the same animals in Fig. 4.

which causes changes in renin secretion. It should be noted that the wide range of creatinine clearances for each animal is to be expected as the GFR of dolphins is quite labile (7).

However, it does appear that the regulation of salt balance by dolphins and pinnipeds may depend in part on an active renin-angiotensin system which stimulates aldosterone secretion. It is possible that aldosterone acts upon renal tubules of these animals in a manner similar to other mammalian species. It remains to the seen, however, what the precise nature of the stimulus is which causes the kidney to alter the secretion rate of renin.

Summary. In both dolphins and sea lions there was found a significant correlation between plasma renin activity (PRA) and the concentration of aldosterone in plasma. Furthermore, an excellent correlation was obtained between the urinary sodium excretion and PRA in two dolphins. These data support the hypothesis that in marine mammals the renin–angiotensin–aldosterone axis plays a role in the regulation of salt balance. However, the signal perceived by the kidney which serves to regulate renin secretion is unknown.

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Effects of Leukocytic Endogenous Mediator on Hemopexin, Transferrin, and Liver Catalase (40118)

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Recent findings have shown that many of the acute phase reactions seen during inflammation or infection can also be produced by injection of leukocytic endogenous mediator (LEM). LEM is a low molecular weight protein that is released from stimulated granulocytes. Recent evidence suggests it is very similar or identical to leukocytic pyrogen (1). It has been found that LEM injection will increase the plasma levels of α_2-macrofetoprotein (2), ceruloplasmin (3), fibrinogen (4), haptoglobin (4), and C-reactive protein (5). All of these are glycoproteins that have an increased rate of synthesis in the liver during inflammation. Other proteins showing changes in response to acute stresses are transferrin, hemopexin, and liver catalase. Transferrin levels decrease after acute injury, which is the result of increased synthesis and an even greater change in the rate of catabolism (6). Likewise, liver catalase decreases following acute injury in this case due to an inhibition of synthesis (7). Less is known concerning the control of hemopexin levels after injury, but it has been suggested that in rats hemopexin acts as a typical acute phase protein (8). This suggestion was based on the finding of increased hemopexin synthesis in response to surgical trauma.

Since transferrin and liver catalase respond to acute stress quite differently from hemopexin and other acute phase proteins, their response to LEM may provide insight as to how LEM influences liver protein synthesis. The specificity of the LEM-induced changes in protein metabolism has not yet been fully established; however, should LEM injection reproduce all aspects of the acute phase response, it must be seriously considered as a possible mediator of the acute phase response. In this study we determined if LEM could simultaneously increase and decrease the synthesis rates of these different proteins as compared to plasma fibrinogen, which is known to increase (4).

Materials and methods. Animals. Holtzman derived rats were maintained at 21° with 12 hr of light and 12 hr of darkness. They were fed Rockland mouse and rat diet and water *ad libitum*. Healthy New Zealand white rabbits weighing 2–4 kg were used for LEM preparation.

Preparation of LEM. Crude LEM was prepared from rabbit peritoneal granulocytes (9) and partially purified by the butanol-methanol method of Rafter *et al.* (10). It was then filtered through a membrane which retained molecules of molecular weight >100,000 daltons (Amicon XM-100) and stored as a dry lyophilized powder. The dose of LEM injeted is expressed in terms of the number of leukocytes from which it was derived in the original preparation.

Determination of hemopexin, fibrinogen, transferrin, and liver catalase. Plasma levels of hemopexin were quantitated by radial immunodiffusion by the method of Mancini *et al.* (11). The monospecific antisera to rat hemopexin was generously donated by Dr. U. Muller-Eberhard, Scripps Clinic and Research Foundation, La Jolla, CA. Fibrinogen was determined on citrated plasma by the heat turbidity method of Wycoff (12). Transferrin was estimated from the total iron binding capacity of the plasma as determined by the method of Schade *et al.* (13). Liver catalase was measured by the method of Bonnichsen *et al.* (14). Statistical analysis was done with the use of Student's paired *t* test.

Results. The effects of injecting varying doses of LEM on hemopexin, fibrinogen, transferrin, and liver catalase in the rat are shown in Table I. The concentration of hemopexin and fibrinogen were increased at the smallest dose tested, and further increases were observed as the amount of injected LEM was increased. Decreases in transferrin and liver catalase occurred only at the highest dose tested. To increase the activity, we did further studies on these two proteins using

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TABLE I. EFFECT OF LEM DOSE ON HEMOPEXIN, FIBRINOGEN, TRANSFERRIN, AND LIVER CATALASE.[a]

Dose of LEM[b]	Hemopexin % of normal	Fibrinogen % of normal	Transferrin % of normal	Liver catalase % of normal
None	100 ± 5[c]	100 ± 12[c]	100 ± 3[c]	100 ± 3[c]
1×10^6	129 ± 11*	132 ± 5*	—	—
1×10^7	152 ± 8***	142 ± 7**	110 ± 2*	99 ± 4
1×10^8	210 ± 12***	197 ± 11***	93 ± 3	104 ± 3
5×10^8	—	—	85 ± 4**	90 ± 4*

[a] Measurements of transferrin were at 12 hr after LEM injections; the other proteins were determined at 24 hr after injection.

[b] Number of rabbit peritoneal granulocytes from which the leukocytic endogenous mediator (LEM) was derived. The partially purified LEM from 10^8 granulocytes had a protein content of approximately 180 μg.

[c] Mean ± SE of eight rats.

* Significantly different from normal $P < 0.05$.

** Significantly different from normal $P < 0.01$.

*** Significantly different from normal $P < 0.001$.

crude LEM before purification. We have no explanation for the apparent increase in transferrrin after injecting a low dose of LEM.

The plasma levels of hemopexin and fibrinogen at various times after LEM injection are shown in Table II. These levels were both significantly increased at 8 hr after injection and remained elevated for at least 24 hr.

Transferrin and liver catalase levels were also affected by a single injection of crude LEM (Table III). When the dose of LEM was expressed per granulocyte, the crude LEM was more active because of the substantial losses in activity during purification. Plasma transferrin levels were decreased significantly at 12 hr and then returned to normal values 24 hr after injection. Liver catalase followed a different time course in that it continued to decrease significantly throughout the 24-hr period after injection.

Further decreases in the level of both transferrin and liver catalase could be demonstrated after a series of injections of crude LEM. Injections of 10^8 LEM were made every 8 hr, and animals were sacrificed 5 hr following the final injection. As shown in Table IV, transferrin and liver catalase were significantly decreased following the fourth injection of LEM.

Discussion. These results confirm and extend earlier reports (1, 2, 4, 5) suggesting that injection of LEM induces many of the "acute phase" reactions that are typical of inflammation or infection. It should be noted that the hemopexin and fibrinogen levels are much more sensitive to LEM injection than are the levels of transferrin and liver catalase.

TABLE II. HEMOPEXIN AND FIBRINOGEN LEVELS IN THE PLASMA OF RATS AT VARYING TIMES AFTER INJECTING LEM.[a]

Time after ip injection (hr)	Hemopexin % of normal	Fibrinogen % of normal
0	100 ± 5[b]	100 ± 12[b]
4	118 ± 8*	109 ± 5
8	153 ± 9***	153 ± 10**
12	160 ± 6***	163 ± 6***
24	210 ± 12***	197 ± 11***

[a] Each rat received an ip injection of partially purified LEM prepared from 10^8 granulocytes dissolved in 1 ml of saline.

[b] Mean ± SE of eight rats.

* Significantly different from normal, $P < 0.05$.

** Significantly different from normal, $P < 0.005$.

*** Significantly different from normal, $P < 0.001$.

TABLE III. TRANSFERRIN AND LIVER CATALASE LEVELS AFTER A SINGLE INJECTION OF 5×10^8 CRUDE LEM.[a]

Hr after injection	No. of animals	Transferrin % of normal	Liver catalase % of normal
0	12	100 ± 2[b]	100 ± 4[b]
12	10	77 ± 1**	89 ± 3*
18	10	84 ± 3**	80 ± 4**
24	10	95 ± 3	75 ± 4**

[a] The dose of LEM is expressed as the number of rabbit peritoneal granulocytes from which the LEM was derived.

[b] Mean ± SE.

* Significantly different from normal, $P < 0.05$.

** Significantly different from normal, $P < 0.001$.

Differences in the amount of LEM required to cause other changes have also been noted. However, once this threshold dose of LEM was exceeded, their respective log-dose response curves were linear. Slope values for LEM-induced changes in fibrinogen levels

E IV. TRANSFERRIN AND LIVER CATALASE .S AFTER MULTIPLE INJECTIONS OF CRUDE LEM.

in- s[a]	No. of animals	Transferrin % of normal	Liver catalase % of normal
	24	100 ± 4[b]	100 ± 3
	10	93 ± 3	103 ± 3
	8	70 ± 5*	55 ± 4*
	10	83 ± 6**	40 ± 3*
	10	65 ± 1*	49 ± 5*

ı 8-hr injection contained LEM derived from ılocytes.
n ± SE.
nificantly different from normal, $P < 0.001$.
nificantly different from normal, $P < 0.05$.

her acute phase reactions have been :d (1).

iously it has been reported that plasma gen responds more quickly to injection M than to injection of endotoxin (4). ırison of the data in Table III with ıed data (15) reveals that transferrin ;ponds faster to LEM injection than to xin injection. Not only is the greatest vident at an earlier time, but the return mal is also much faster. This time is compatible with the suggestion that nay be a mediator of the acute phase se.

:iple injections of LEM appeared to be :ffective than a single large dose. It ikely during inflammation or infection nall amounts of LEM would be re- from the granulocytes over a long of time. Thus the multiple injections umed to more nearly approximate the release of LEM.

finding that LEM injection could pro- evations in the plasma levels of hem- and fibrinogen and at the same time lepression in the levels of transferrin r catalase indicates it has a specific n in the acute phase reaction. Previous ;ations with an *in vitro* liver slice sys- ve indicated that LEM does not act on the liver to produce its effect on ;en synthesis but may act to release an :diate which does stimulate *in vitro* ;en synthesis (4). Further studies are needed to determine if the same chain of events is responsible for changes in the levels of other proteins.

Summary. Elevations in the plasma levels of hemopexin and fibrinogen were found following injection of leukocytic endogenous mediator (LEM) into rats. Liver catalase and plasma transferrin were decreased following injection of LEM. Greater amounts of LEM were required to depress liver catalase and plasma transferrin than were required to elevate hemopexin or fibrinogen. Multiple injections of LEM at 8-hr intervals were found to be more effective for depression of liver catalase and plasma transferrin than a single large dose of LEM.

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Cigarette Smoking Baboon Model: Demonstration of Feasibility (40119)

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To investigate the mechanisms by which cigarette smoking affects atherosclerosis, chronic obstructive pulmonary disease, and fetal development, an animal model is required that meets several criteria. Ideally, the animal model should simulate human cigarette smoking in puff frequency, duration, and volume because puff characteristics affect smoke composition. The animal also should inhale through the mouth because nasal passages modify the smoke. The exposure system should avoid surgical manipulation or stressful restraint during smoke exposure because these procedures may affect physiological endpoints. The animal also should be susceptible to diet-induced atherosclerosis, and its respiratory and reproductive systems should be similar to those of man.

Most animal models in current use for cigarette smoke inhalation studies do not meet all of these criteria. These include small rodents exposed to cigarette smoke in chambers (1), rabbits exposed in chambers (2) or through face masks (3), dogs exposed through tracheostomata (4) or face masks (5), chickens exposed through face masks (6), and nonhuman primates exposed through face masks (7, 8). Preliminary experience with a few baboons (9) and the reported experiences of other investigators in training nonhuman primates to smoke (10–13) led us to believe we could train a substantial number of baboons to smoke and could control their smoking behavior. The present report describes a system which has accomplished this objective.

Methods and materials. Training methods. Seven baboons (*Papio cynocephalus*), ranging from 1 to 4 years in age, were given water from a handheld water bottle until they learned to suck rather than to lick water. Then a Smoking Inhalation Response Indicator and Conditioner (SIRIC) (14), a device delivering a measured water reward when the animal's puff exceeded investigator-controlled criteria, was mounted on each animal's cage. Smoke from the burning cigarette passed to the animal through a 6 cm tube which served as a cigarette holder and as a mouthpiece. To teach the animal to suck on the cigarette holder, the trainer squirted water through the cigarette holder of the SIRIC and blocked the end of the tube with his finger. When the animal sucked water from the tube with a negative pressure greater than 1.24 cm H_2O for a minimum of 0.25 sec, a water reward was delivered through a separate drinking tube.

After the animal had learned to activate the SIRIC and consume the water reward, the opening was left blocked with an unlighted cigarette so that the animal could activate the device at will. Within 25 days all animals learned to earn their entire water ration by activating the SIRIC. Then lighted cigarettes were presented, and the animals readily learned to make 0.25 sec puffs of cigarette smoke in order to earn their water. An attendant then presented lighted cigarettes at regular intervals (10, 15, 20, or 30 minutes) during 8 hr per day, 7 days per week. The minimum puff duration was increased gradually from 0.25 to 2.50 sec over 1–9 months (mean, 121 days).

Criterion for smoke inhalation. Carbon monoxide (CO) concentration in the blood was used as the criterion for smoke inhalation. CO is absorbed only slightly in the buccal cavity, but is readily absorbed in the lungs (15).

Venous blood samples were drawn under ketamine (Ketaset, Bristol Laboratories, Syracuse, NY 13201) sedation within 10–20 min after the animals had smoked the last of a series of cigarettes over a 3-hr period. The samples were analyzed for CO by the method of Ayres *et al.* (16) with a modified extraction

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ıber; CO is extracted from whole blood then measured by gas chromatography ; a thermal conductivity detector.

; the criterion for smoking, we selected a entration of CO in blood of 0.30 ml/dl, : times the mean (0.10 ml/dl) and twice naximum (0.15 ml/dl) of the values ob- :d in the seven animals prior to cigarette :e exposure (range 0.05–0.15 ml/dl). We ıated the maximum amount of CO that ıon blood can carry by saturating 12 d samples from smoking and nonsmok- ınimals with CO and measuring the CO entrations. The four samples with the est concentrations averaged 14.68 ml dl.

:perimental cigarettes. We conducted of the training using University of Ken- y Reference (UKR) cigarettes (17, 18); also were conducted with Code 5 and : 14 experimental cigarettes (Series 1) of ;moking and Health Program of the Na- ıl Cancer Institute (19). Selected charac- :ics of these cigarettes are given in Table

:sults. Training. The average time re- :d to train the seven baboons to smoke cigarettes was 96 days (Table II); the minimum was 32 days and the maximum, 178 days. These durations were measured from the first day a trainer worked with the animal to the first day on which the blood CO concentration equaled or exceeded 0.30 ml/dl. Figure 1 illustrates acquisition of the cigarette smoking response and inhalation of smoke as measured by mean blood CO concentration. CO levels in week 8 were significantly higher than in week 2, and were significantly higher in week 14 than in week 8.

Maintenance of smoking behavior. Once the animals were trained to smoke cigarettes for water rewards, no difficulty was encountered in maintaining or controlling the smoking behavior. Two animals performed without interruption for well over a year, earning all of their water by smoking cigarettes. The animals remained in good health throughout the period of training and testing of the system and gained weight at a rate normal for animals of their age. Hematocrits measured weekly for 1 month were within the normal range and showed no unusual fluctuations. No significant correlations were observed between hematocrit and total water delivered per day or ml water delivered per kg body weight.

In the six smoking animals examined weekly for 4 months, the average COHb was 5.9% (0.89 ml CO/dl); the maximum mean value was 16.0% COHb (2.40 ml/dl).

Variables affecting smoking behavior. After smoking behavior was established for six animals, various combinations of cigarettes, puff duration settings, cigarette frequency, ratio of reinforcement, and amount of water reward were tested for their effects on smoking behavior. The animals readily accepted Code 5 and Code 14 cigarettes. Puff duration

LE I. SELECTED CHARACTERISTICS OF SMOKE RODUCED BY EXPERIMENTAL CIGARETTES.

	Cigarette type		
Characteristics	UKR	Code 5	Code 14
particulate matter)	38.5	28.7	14.1
ıg)	35.8	24.2	11.3
ıe (mg)	2.63	1.65	0.20
ıl)	17.6	14.5	11.9
	10.9	10.2	5.9

umber of puffs required to produce a butt of 23 ıder standard smoking machine conditions (16) of puffs of 2 sec duration at a rate of one per minute.

BLE II. AGE, SEX, DAYS TO CRITERION, AND MONTHS SMOKING BEHAVIOR MAINTAINED FOR SEVEN BABOONS.

ıboon number	Age at start of training, months	Sex	Days to criterion	Months smoking maintained
B-734	32	Male	94	15
B-756	30	Male	134	14
B-941	9	Female	94	0.5[a]
B-944	9	Female	101	8
B-720	43	Male	178	2
B-778	40	Male	32	5
B-966	21	Male	40	3

ıimal died of unexplained causes.

required for a reward was advanced to as long as 3.0 sec.

Since the animals' primary incentive for smoking was the water reward, it was possible to control the number of puffs per day by adjusting the reinforcement contingency. For example, when the water reward per criterion puff was changed from 1.00 ml to 0.75 ml, a 25% reduction, the animals' mean number of puffs per day rose from 467 (SEM = 53) to 570 (SEM = 52), an increase of 22%. The mean water earned per day remained essentially constant, decreasing from 432 ml (SEM = 42) to 417 ml (SEM = 48), and puff duration requirements remained constant over the two test weeks. Other manipulations of basic operating conditions produced equally orderly changes in the animals' smoking behavior.

Variables affecting blood CO concentration. Although we did not perform controlled experiments to isolate the effects of single, investigator-controlled variables on blood CO during the feasibility testing period, it appeared that this measure of inhalation was affected by type of cigarette smoked as well as by other variables controlled by the tigator (Table III). There was a th difference between blood CO concen in the five animals while smoking C cigarettes as compared to the blood C(smoking UKR cigarettes. To make an alent number of puffs per 3 hr for ea(it was necessary to present more C cigarettes because they are loosely and burn rapidly.

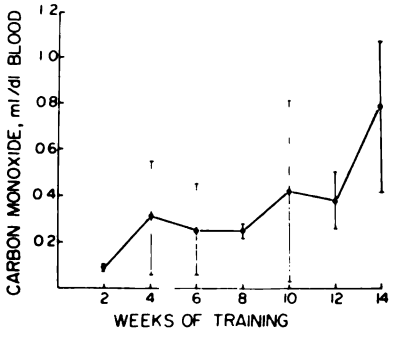

FIG. 1. Mean and 95% confidence intervals of concentration of carbon monoxide in blood of six baboons by week of training.

Use of model in controlled experime feasibility, demonstrated here, of us baboon as a model to study the ef cigarette smoke inhalation was furth firmed when, subsequent to the d mental work, we trained an additi baboons to smoke with the SIRIC countered no training failures. We al extended the total daily smoking exp 12 hr and 48 cigarettes per day v difficulty. One attendant can establi maintain smoking behavior in approx 20 baboons. Control animals can be su to identical training and maintenanc dures by using a filter with the appr draw resistance of a cigarette as a sha

Discussion. Comparison of human boon smokers. Studies of human s have shown mean COHb saturation v 4.4% (20); 4.7% (21); and 6.7% (22). C of human smokers had COHb sat equal to or greater than 14% saturatio blood CO levels of 5.9% COHb are si those shown by human smokers and maintained for long periods in the c smoking baboon.

Moody and associates (27) found human cigarette smoker made, on th age, 9.5 puffs per cigarette on 21.6 ci per day, giving an average "dose" puffs of cigarette smoke per day. O final 17 week period of this study th

TABLE III. CIGARETTES PRESENTED, PUFF DURATION, NUMBER OF PUFFS, AND BLOOD CARBON M(CONCENTRATION IN FIVE BABOONS AFTER 3 HR OF SMOKING TWO TYPES OF CIGARETTES, MEA MEASUREMENTS 1 DAY PER WEEK FOR 5 WEEKS.

	Type of cigarette					
	UKR		Code 14			
Variable	Mean	SEM	Mean	SEM	*df*	*t*
Cigarettes presented, number	8.7	0.34	23.3	0.32	—	—
Minimum puff duration, sec	1.66	0.28	2.18	0.26	4	3.90
Puffs, number/3 hr	238.0	44.2	260.0	37.5	4	1.60
Carbon monoxide in blood, ml/dl	0.48	0.06	1.50	0.27	4	4.75

ıveraged 535 puffs per day. The mean ıration for humans (27) was 2.3 sec; ɔoons' average puff duration was 2.0 e system described here allows consimulation of the general features of cigarette smoke exposure with the . Furthermore, because cigarette appears to lack strong appetitive or ɔ properties in the baboon, the invesscan control any specified degree of e smoke inhalation by manipulating riate variables.

rence in blood CO between cigarettes.) yield from UKR cigarettes on stanıoking machines is 1.5 times that from 4 cigarettes (23). CO yields of comcigarettes vary by factors of two to 4–26), but CO levels in smokers vary rably more, probably because of difs in puffing characteristics such as puff and duration (25).

ɔ 14 cigarettes have a lower draw re- than UKR cigarettes (5.05 cm H_2O e drop vs 7.07). Therefore, for the ɔ to achieve negative pressure suffi- activate the SIRIC, they had to gen- larger puff volume. In addition, the mean puff duration requirement when mals were smoking Code 14 cigarettes c vs 1.66 sec) also contributed to larger ɔlume and deeper inhalation. CO is t to be responsible for some of the ious effects of cigarette smoking. The es affecting CO absorption can be vith this system, since the investigator ıtrol each of the conditions affecting characteristics.

ılness of the baboon smoking model. boon is suitable for the investigation ·ette smoking effects on experimental clerosis (28, 29) and reproductive ogy (30). Its large size facilitates per- tests of pulmonary function. The ed instrumentation and operant techpermit controlled experiments on the ɔf cigarette smoke inhalation with adnumbers of subjects. Since we have tered no training failures, it is possible n animals randomly to cigarettes or to Investigator control of smoking perce reduces potential confounding by ıabituation or avoidance by the anihe short smoke delivery path minimizes changes in smoke composition due either to removal of smoke components or to particle agglomeration. The demonstration of elevated blood CO concentrations confirms delivery of cigarette smoke to the lower respiratory tract.

Summary. Using operant conditioning techniques and a specially designed apparatus, and without surgical manipulation or obvious stress, we trained seven baboons to puff on lighted cigarettes and to inhale cigarette smoke. Smoking behavior was maintained for up to 15 months and could be controlled by manipulating minimum puff duration, water reward, and frequency of presenting cigarettes. Concentrations of blood carbon monoxide after 3 hr of smoking equaled or exceeded those reported for heavy human cigarette smokers. The cigarette smoking baboon models human smoking performance and is a useful animal model for studying the mechanism of the effects of cigarette smoke inhalation on the cardiovascular, pulmonary, and reproductive systems.

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Inhibition of Prostaglandin-Induced Pulmonary Vasoconstriction by Organic Acid Transport Inhibitors (40120)[1]

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Prostaglandins (PGs) are largely inactivated upon a single passage through the pulmonary circulation (1). Movement of PGs into the cell is required for metabolism of these compounds (2, 3). Several investigators (4–7) have presented evidence supporting the concept of carrier mediated transport of PGs into various tissues including lung. For example, Bito and Baroody (5) demonstrated that $PGF_{2\alpha}$ uptake and metabolism by the perfused rat lung could be largely blocked with the organic acid transport inhibitors probenecid and bromcresol green (GBr). Bakhle *et al.* (8) made similar observations with PGE_2 in the presence of BGr or thymol blue (0.01 m*M*). However, no studies have been made of the consequence of such inhibition by organic acid transport inhibitors on the hemodynamic actions of these PGs. Using the isolated perfused canine lung lobe, we have explored the possibility that probenecid and bromcresol green could modify the previously demonstrated pulmonary pressor activity of the bisenoic PG precursor arachidonic acid (AA) (9), a synthetic PG endoperoxide analogue (10, 11), and $PGF_{2\alpha}$ (12). Norepinephrine was used to test the reactivity of the preparation and also to ascertain whether probenecid or BGr inhibited the pulmonary pressor response to this catecholamine.

Materials and methods. Mongrel dogs (11–18 kg) of either sex and unknown age were anesthetized with sodium pentobarbital (30 mg/kg iv). The left lower lobe of the lung was perfused as described previously (9). Autologous citrated blood was pumped at constant flow rate into the cannulated lobar artery and was collected from the lobar vein into a reservoir to be recycled. The lung was ventilated with ambient air.

The sodium salt of arachidonic acid (5,8,-11,14-eicosatetraenoic, 99% pure; NuChek Prep. Inc., Elysian, MN) was prepared daily by dissolving in sodium carbonate (100 m*M*) with constant stirring under nitrogen in the absence of light. The resulting solution (10 mg/ml) was used only if clear. The endoperoxide analogue used in this study was obtained from The Upjohn Co., (Kalamazoo, MI): (15s)-hydroxy-11α, 9α epoxymethano prosta 5Z, 13E-dienoic acid, (abbreviated 11–9 EM) was prepared daily by diluting stock solution with saline to a final concentration of 20 μg/ml. The tromethamine salt of $PGF_{2\alpha}$ (The Upjohn Co.) was prepared in saline (100 μg/ml) from stock ethanol solutions. Norepinephrine (NE; Levophed-Winthrop Laboratories, New York, N.Y.) was diluted in saline (100 μg/ml) just prior to use. Probenecid (Merck, Sharp and Dohme Laboratories, Rahway, NJ) was prepared by dissolving in NaOH (2 *N*). The pH was adjusted to 7.4 with monopotassium phosphate buffer to a final concentration of 10 mg/ml. Bromcresol green (Fisher Scientific, Springfield, NJ) was prepared similarly except that it was dissolved in NaOH (1 *N*) and the final concentration was 1 mg/ml. Indomethacin (Merck, Sharp and Dohme) was dissolved in an aqueous sodium carbonate solution to a final concentration of 100 mg/ml.

Pressor agents were administered as bolus injections directly into the inflow cannula just proximal to the point of its insertion in the lobar artery. Responses to these substances were recorded for the following doses (μg/kg): AA, 100; 11–9 EM analogue, 0.5; $PGF_{2\alpha}$, 1; and NE, 1. The inhibitors were added directly to the reservoir and were circulated through the isolated lobe to allow equilibration before being challenged with the pressor agents. The final concentrations (μg/ml) of the inhibitors used in these experiments were as follows: indomethacin, 100;

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probenecid, 425 and 850, and BGr, 6, 12 and 24. The pressor agents, with the exception of 11-9 EM, were administered in the presence of each inhibitor at the concentrations indicated. The order of administration of these agents was randomized for each concentration of inhibitor. The 11-9 EM was given only in the presence of the highest concentration of each inhibitor. Vascular responses to the pressor substances were reported as mean percentage changes from control perfusion pressure ± SEM. Results were analyzed with Students' t test, and differences were considered significant if $P < 0.05$.

Results. Administration of each inhibitor resulted in a transient 0–2 mm Hg drop in baseline perfusion pressure which returned to control before pressor agents were injected. The results of experiments conducted with probenecid are summarized in Fig. 1A. The mean control pulmonary pressor response to AA of 66 ± 9% was reduced significantly to 33 ± 8% by probenecid (425 μg/ml). The response to AA was not attenuated further by higher concentrations of probenecid. Like AA, the pulmonary pressor response to $PGF_{2\alpha}$ was diminished significantly from 45 ± 9% to 20 ± 10% by probenecid (425 μg/ml). The $PGF_{2\alpha}$ pressor response was almost completely inhibited by higher concentrations of probenecid (850 μg/ml). The mean control pulmonary pressor response to 11-9 EM of 156 ± 24% was not inhibited significantly (125 ± 41%) by probenecid up to 850 μg/ml. In six animals, the mean control pulmonary pressor response to NE was 36 ± 4%. This response was reduced significantly by probenecid at only the higher dose (850 μg/ml).

The effects of BGr as an inhibitor of the pulmonary pressor activity of these four compounds are summarized in Fig. 1B. In contrast to probenecid, the response to AA was not reduced significantly (66 ± 9% vs. 57 ± 11%) by BGr (24 μg/ml). In five experiments the mean response to 11-9 EM (164 ± 26%) was not inhibited significantly by BGr (24 μg/ml).

The response to $PGF_{2\alpha}$ was diminished significantly from a mean of 57 ± 7% to a mean of 31 ± 9% by BGr (6 μg/ml). This inhibitory action was not augmented appreciably by higher concentrations of BGr. At a 12 μg/ml of BGr, the pressor response to $PGF_{2\alpha}$ was 29 ± 7% and was virtually unchanged at 24 μg/ml of BGr.

The pulmonary pressor response to NE was not reduced significantly by BGr at the highest concentration used. As anticipated, indomethacin blocked the pulmonary pressor response to AA in every instance ($n = 5$). It

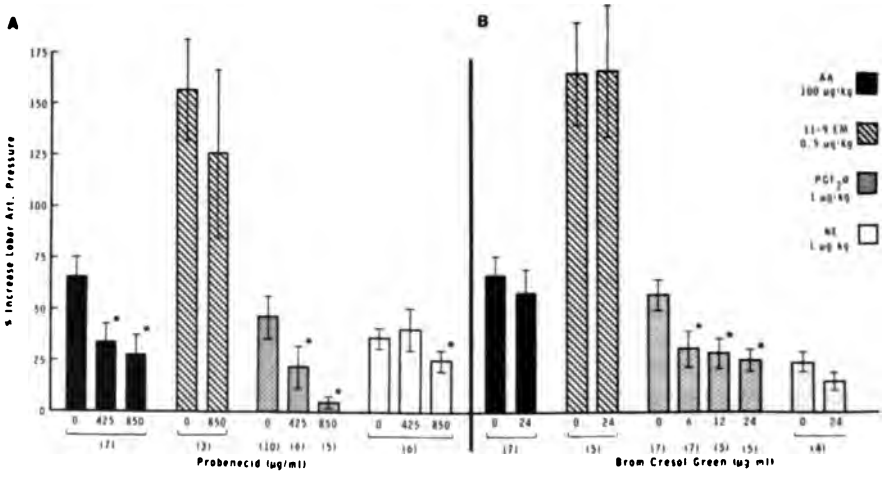

FIG. 1. The pulmonary vasopressor responses to arachidonic acid (AA), the PG endoperoxide analogue (11-9 EM), $PGF_{2\alpha}$, and norepinephrine (NE) in the absence and presence of probenecid (Fig. 1A) and bromcresol green (Fig. 1B). Each inhibitor alone did not change the baseline perfusion pressure (14–15 mm Hg). The dose of each pressor agent is shown to the right of the second panel and the concentration of the inhibitor is shown below each bar. The SEM is illustrated on each bar and the number of observations (n) is indicated below each group of bars. The asterisks indicate a significant difference ($P < 0.05$) from each control pressor response.

ed a slight (20%), though not significant ıncement of the 11-9 EM pressor action 3). The responses to $PGF_{2\alpha}$ and NE (*n* each) were not altered after indometha-

iscussion. The pulmonary pressor effects ıA and $PGF_{2\alpha}$ were inhibited by pro-ıcid, a clinically useful organic acid trans- inhibitor. At the highest concentration, enecid inhibited the action of NE, but not reduce the pulmonary pressor re-se to the 11-9 EM.

ıe pulmonary pressor effect of AA is due ısoactive products formed from AA since ɔlocked by PG synthetase inhibitors such ;pirin (9). The vasoactive products of AA ıde endoperoxides, PGs (13), and throm-ınes (14). Probenecid has no PG synthe- inhibitory properties (15), therefore the gonism of AA action by probenecid is lo a mechanism other than PG synthetase ɔition. One possible mechanism is the ɔition of uptake of AA into the cell, enting the conversion of AA into active ucts. Probenecid is a competitive organic transport inhibitor in the kidney, and been described as a PG transport inhibi-n the rat lung (5), chicken and rat kidney 6) and other tissues (4). A second possible ıanism is that probenecid may interfere the release of vasoactive products of AA ı the cells. Hamberg and others (13) have ′n that a large fraction of the products ed in the lung following injection of AA eleased into the circulation, whereupon may act at downstream sites. A third ible mechanism, that probenecid may act :tly as a competitive inhibitor of the pul-ary pressor products formed from AA, explored by examining the effects of enecid on responses to the endoperoxide ɔgue and $PGF_{2\alpha}$.

ıe naturally occurring endoperoxides $ɔ_2$ and PGH_2 are unstable and are not rally available in sufficient quantities for : in dogs. The stable endoperoxide ana-e 11-9 EM (17) has been found to share : pulmonary vasoconstrictor and airway ɔnses with the natural endoperoxides. $ɔ_2$ and PGH_2 constrict feline pulmonary y and increase airway resistance in the ea pig (18); the PGH_2 analogue increases ıonary artery pressure (10) and airway resistance (19) in the dog. Probenecid did not inhibit the action of the 11-9 EM, indicating that the analogue has access to its site of action.

The pulmonary pressor response to $PGF_{2\alpha}$ was blocked in a dose related manner by probenecid. Since most evidence from receptor binding studies point to the cell membrane as the site of action of $PGF_{2\alpha}$, uptake into the cell may not be necessary for $PGF_{2\alpha}$ action. Therefore, probenecid may inhibit $PGF_{2\alpha}$ action at the cell membrane, but the present data are not sufficient to determine whether this inhibition is competitive.

Since Bito and Baroody (5) reported that probenecid inhibits the PG catabolizing enzyme 15-hydroxy PG dehydrogenase in the rat lung, an enhancement of $PGF_{2\alpha}$ action in the dog lung was expected. Instead, an inhibition of its pressor action was observed. It is very unlikely that the dehydrogenase activity is enhanced by probenecid in the dog lung, therefore the inhibition of $PGF_{2\alpha}$ action by probenecid is independent of the PG catabolizing enzymes.

Thus, the mechanism by which probenecid inhibits the effect of AA in the lung remains unclear. Although $PGF_{2\alpha}$ action is blocked, this explains only part of the inhibition since Hamberg *et al.* (13) reported that $PGF_{2\alpha}$ compromises only a small fraction (<6%) of the products of exogenous AA released from the perfused guinea pig lung. Other enzymes or vasoactive products of the AA metabolic pathway may be affected by probenecid.

The pulmonary pressor response to NE was inhibited significantly only at the highest concentration of probenecid. This observation demonstrates that the pulmonary vasculature was still reactive to a catecholamine at probenecid levels that inhibited AA and $PGF_{2\alpha}$.

BGr inhibited only the pulmonary pressor response to $PGF_{2\alpha}$. This indicator dye achieved approximately 50% inhibition at only 6 μg/ml. At comparable concentration of BGr, Bito and Baroody (5) found a 95.7% inhibition of $PGF_{2\alpha}$ uptake in the perfused rat lung. Perhaps BGr at this concentration of 6 μg/ml is interfering maximally with the response to $PGF_{2\alpha}$, although not causing complete inhibition. Since nearly complete inhibition of $PGF_{2\alpha}$ can be achieved with

probenecid and not with BGr, and AA is inhibited by probenecid and not by BGr, these substances may act in different manners that are unrelated.

Summary. Probenecid and bromcresol green (BGr), reported to be PG transport inhibitors, were tested for their effectiveness in modifying the vasopressor responses of arachidonic acid (AA), an endoperoxide analogue, $PGF_{2\alpha}$ and norepinephrine in the isolated perfused canine lung lobe. Only the pulmonary pressor action of AA was blocked by pretreatment with indomethacin. Probenecid attenuated AA responses and blocked those of $PGF_{2\alpha}$ in a dose-related manner. BGr had no antagonistic activity against AA and inhibited $PGF_{2\alpha}$ action by 50%. Probenecid and BGr appear to inhibit $PGF_{2\alpha}$ differently. Neither blocked the pressor action of the endoperoxide analogue, and neither attenuated the norepinephrine pressor action at PG inhibiting concentrations. The use of organic acid transport inhibitors represents a potentially useful approach in studying the mechanisms of action of AA and its metabolic products.

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Effects of Carbon Monoxide on Isolated Heart Muscle (40121)

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The mechanism by which carbon monoxide (CO) affects the mammalian organism was first described by Haldane (1) and has since been confirmed by numerous other workers (2, 3). Briefly, CO binds with blood hemoglobin to form carboxyhemoglobin. This causes a functional anemia by decreasing the amount of hemoglobin available for oxygen transport and by causing the oxyhemoglobin dissociation curve to shift to the left. If the tissue hypoxia is severe enough, the resultant of these two effects can lead to a fatal event.

There are, however, several reports in the literature suggesting that CO might have a direct effect on functioning tissue in addition to its hemoglobin-mediated effects (4–7). The following experiments were undertaken to study the effects of CO on the isolated, rat right ventricle preparation contracting in a hemoglobin-free medium.

Methods. Male, white rats weighing 312 ± 14 g were guillotined and the hearts excised rapidly. The right ventricle was dissected away and split into two pieces. One strip was mounted on an electrode-bearing block, attached to a strain gauge transducer and immersed in an oxygenated Krebs–Ringer solution as previously described (8). The preparation was stimulated at a rate of 60 per min and resting and developed tensions were recorded. At the end of one hour the aerating gas mixture of 95% O_2–5% CO_2 was changed to either 95% N_2–5% CO_2 (N_2-stress) or 95% CO-5% CO_2 (CO-stress). After 10 min of the stress gas, oxygen was again bubbled through the medium and the contracting muscle was allowed to recover for 10 min in 95% O_2–5% CO_2 (normoxia) or 70% O_2–25% CO–5% CO_2 (CO-hypoxia).

The time in minutes required for the contracting muscle to decline to one half of its preanoxic amplitude during the N_2 or CO-stress was determined and expressed as T_{50}. The change in resting tension in grams at the end of 5 min of stress was expressed as $\Delta T_5'$. The extent of recovery after 10 minutes of normoxia or CO-hypoxia was determined by comparing the ratio of the recovery amplitude to the pre-anoxic amplitude and expressing the results as percent recovery (%R) according to the following formula (9).

$$\%R = \text{Recovery amplitude} / \text{Preanoxic amplitude} \times 100$$

The Student t test was used to test the significance of differences between means (10). Tissue electrolytes were also determined on nitric acid digests by atomic absorption spectrophotometry according to the method of McGrath and Bullard (8). Briefly, one portion of the right ventricle was prepared for analysis immediately while the second portion was prepared after recovery from the N_2- or CO-stress. The tissues were dried overnight in an oven at 100°, digested with nitric acid and analyzed for sodium and potassium. The results were expressed as percent increase or decrease from control values.

Results. Developed tension decreased and resting tension increased in the isolated right ventricles during either N_2- or CO-stress. The time required for the developed tension to decrease to one half of its pre-stress value (T_{50}) was 2.5 minutes in N_2 and 2.3 min in CO (Table I). The difference between these values is not significant. The change in resting tension (ΔT) at the end of 5 minutes of stress was 1.0 g in nitrogen and 0.9 g in CO. The difference between these values is not significant.

The percent recovery (%R) after 10 min of normoxia was 43.5 % in nitrogen and 63.0% in CO (Table II). Recovery was significantly greater in the CO-stressed muscles. Percent recovery was reduced when the N_2-stressed or the CO-stressed muscles were allowed to recover in CO-hypoxia. Under these conditions recovery was reduced to 31.6% and

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36.9% in the N_2 and CO-stressed muscles, respectively. The difference between these values is no longer significant.

After the N_2- and CO-stressed muscles had recovered in oxygen, the electrolyte content was determined and compared to pre-stress levels. With both gas stresses, sodium levels were increased and potassium levels were decreased at the end of the recovery period (Table III). Similar electrolyte changes were noted in the N_2- and CO-stressed muscles that recovered with CO present in the recovery mixture.

Discussion. We have demonstrated a direct effect of carbon monoxide on functioning heart muscle. The effect of CO on heart muscle was similar in some ways to that of nitrogen in that it caused a reduction in developed tension, an increase in resting t and a reduction in percent recovery. ever, we were surprised to observe th effect was also different in that th treated muscle appeared to be somewh tected during the gas stress and that covered to a significantly greater degre oxygen was reintroduced into the b medium. There is evidence in the lit that CO can be burned by living tissue and Cobb (11) showed that the stim action of CO on the respiration of fr rat skeletal and cardiac muscle was oxidation of CO to CO_2. This work w firmed by Schmitt and Scott (12) and nard (13). Clark et al (14) working wi tissue showed that the rate of CO oxi was greatest for heart muscle, next for s muscle and negligible in skin and Clark (15) also demonstrated CO-bur intact turtles and mice.

If these results can be applied to ou ent study, it is possible that our CO-s muscles were able to burn a small amo CO under the conditions of the expe and that this somehow ameliorated its effects. It is not known what the mec underlying this protective effect is or might act to maintain tissue integrity tissue recovery following the CO-stre more complete. However, if tissue in was better maintained during CO-stre tissue would be less damaged and wh ygen was again made available capab greater degree of recovery. That tissue rity was affected by both gas stresses is by the electrolyte changes that occurre sodium content of both the N_2- an stressed muscle increased, while the sium content decreased. The results dium and potassium are similar to th ported by others (16, 17) and seen b earlier studies (18). However, it is not p from the techniques used in this st

TABLE I. TIME TO 50% CONTRACTION AMPLITUDE (T_{50}) AND CHANGE IN RESTING TENSION AT 5 MIN ($\Delta T_5'$) DURING (A) NITROGEN OR (B) CARBON MONOXIDE-INDUCED ANOXIA.

Treatment	*n*	T_{50} (min)	$\Delta T_5'$ (+g)
A 95% N_2-5% CO_2	(12)	2.5 ±.2	+1.0 ±.1
B 95% CO-5% CO_2	(12)	2.3[a] ±.2	+.9[a] ±.2

[a] = N.S. Values are means ± SE. Student *t* test used to test significance.

TABLE II. PERCENT RECOVERY (%R) IN OXYGEN OR CO-HYPOXIA AFTER (A) NITROGEN OR (B) CARBON MONOXIDE-INDUCED ANOXIA.

	Oxygen		CO-hypoxia	
Treatment	*N*	*%R*	*n*	*%R*
A 95% N_2–5% CO_2	(12)	43.5 ±4.0	(14)	31.6 ±5.8
B 95% CO–5% CO_2	(12)	63.0* ±4.3	(14)	36.9[a] ±7.5

[a] = N.S. Values are means ± SE. Student *t* test used to test significance. Number of hearts per group is given in parenthesis.

* $P < 0.05$.

TABLE III. TISSUE ELECTROLYTE CHANGES AFTER RECOVERY IN OXYGEN OR CO-HYPOXIA AFTER NITROGEN OR (B) CARBON MONOXIDE-INDUCED ANOXIA.

	n	Na	n	K
I. Recovery in oxygen				
A 95% N_2–5% CO_2	(10)	+23%	(11)	−18
B 95% CO–5% CO_2	(11)	+21%	(12)	−17
II. Recovery in CO				
A 95% N_2–5% CO_2	(11)	+19%	(12)	−24
B 95% CO–5% CO_2	(10)	+16%	(12)	−24

nine if cellular disruption was more
ie in the N_2-stressed muscle.
:h the N_2-stressed and CO-stressed mus-
ecovered somewhat more erratically
recovery occurred in the oxygen-CO
ire. First, the extent of recovery was
ed in both cases so there was no longer
lifference in *%R*. Second, the range of
vas always greater when recovery oc-
d in the CO mixture, and with both
es there were two experiments in which
eparations failed to recover completely.
variability of *%R* was always less and
was always some degree of recovery
recovery occurred in oxygen.
eral workers have shown that CO stim-
respiration and Breckenridge (19) dem-
ated that carbon monoxide oxidation is
zed by cytochrome oxidase in heart
le fraction. Thus, the depressed recovery
action in our CO-hypoxia experiments
be due to the increased O_2 demand
d on the muscle during recovery when
vailable oxygen supply was marginal. It
ssible that during recovery in oxygen,
oxygen was available for restitution of
ll and normal metabolic activity, while
g recovery in CO available O_2 was di-
between these two activities and a CO-
lated component. Thus, function as ex-
ed by *%R* would be reduced when CO
resent in the recovery mixture.

mmary. The direct effects of carbon
xide and nitrogen-induced anoxia on
solated isometrically-contracting right
icle of the rat were assessed and com-
. Both gas stresses caused a decrease in
oped tension, an increase in resting ten-
and a reduction in percent recovery
the preparations were reoxygenated.
was no significant difference in the
ie in developed tension or the increase
sting tension between the two groups.
very after 10 min of reoxygenation was
icantly greater in the CO-stressed mus-
Recovery from either anoxia was se-
verely depressed when CO was present dur-
ing the recovery phase.

Tissue sodium levels were elevated while potassium levels were depressed by both stresses.

These results indicate that CO may have a direct effect on the heart in addition to its well known hemoglobin mediated effect.

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Blockade of Mitogen Induction of the Interferon Lymphokine by a Phenolic Foo Additive Metabolite[1] (40122)

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The *in vitro* immune response results from a complex sequence of events involving B- and T-lymphocytes and macrophages (1-3). We have recently demonstrated that a phenolic compound, gallic acid (GA) (3,4,5-trihydroxybenzoic acid), suppresses the *in vitro* plaque-forming cell (PFC) response by directly inhibiting a macrophage function required for T-lymphocyte participation in the immune response (4, 5). This has been demonstrated by the following observations: (a) The *in vitro* PFC response to *E. coli* 0127 lipopolysaccharide (T-independent antigen) by spleen cells from athymic nude mice, which lack functional T-lymphocytes, was unaffected by concentrations of GA that blocked the anti-sheep red blood cell (SRBC) response (T-dependent) in C57B1/6 mice (5), and (b) adherent cells, adherent cell supernatant fluids, and 2-mercaptoethanol (2-ME) reversed GA-induced suppression of the PFC response (4, 5).

The effects of GA on the immune response are of particular interest since GA is both an additive metabolite of propyl gallate and tannic acid, and a normal constituent of food. Large amounts are consumed as free GA and as hydrolyzeable tannins in foods such as tea, cocoa, and coffee (6). Further, tea tannins have been causally linked to hepatic and esophageal cancers (7, 8).

Another measure of T-lymphocyte function which might be influenced by GA through its action on macrophages is lymphokine production. The ability of the T-lymphocyte mitogens concanavalin A, phytohemagglutinin, and staphylococcal enterotoxin A (SEA) to suppress the *in vitro* response of mouse spleen cells to SRB shown to be proportional to their abi induce lymphokine interferon (immune feron) in the cultures (9). Further, di yladenosine 3',5'-cyclic monopho (cAMP) blocked both mitogen induct suppressor cell activity and producti immune interferon in a manner tha gested a direct relationship between t (10, 11).

GA was examined for its effect c induction and action of the quantifiabl phokine, immune interferon. Because feron induction is both quantitatively r to suppressor cell activity (11) and dep on the presence of functional macro (12), it is a logical assay system for stu the immunobiological effects of GA other environmentally encountered pounds that potentially interfere with n immunoregulation.

Methods and materials. Animals. C5 female mice, 8- to 10-week-old, were ob from Jackson Laboratories, Bar Harbo

Antigens. SRBC were obtained fron orado Serum Co., Denver, CO. Culture immunized *in vitro* with 3×10^6 SRBC

Cultures. Dissociated mouse spleen were cultured exactly as described by M and Dutton (13). Direct PFC assays performed on microscope slides (14) a days of incubation.

Reagents. SEA was produced by th crobial Biochemistry Branch, Division crobiology, Food and Drug Administr Cincinnati, OH. Its purity was estima be >99% by extinction coefficient (15 was obtained from ICN Pharmaceu Cleveland, OH, and added to cultu culture medium. The percentage of c and hydrogen and the melting point ra the GA used in these experiments agre literature values. The 2 ME, 99% pur

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l from Matheson, Coleman and Bell, ıd, OH, and added to designated cul- a final concentration of 5×10^{-5} *M*. *Thymidine incorporation.* DNA syn- ras determined by the addition of 1 -thymidine (New England Nuclear, MA) to Mishell-Dutton cultures for l 18 hr of a 72 hr incubation period. ere harvested on glass fiber pads by filtration, washed twice with normal nd once with trichloroacetic acid. The re placed in liquid scintillation vials ml of Dimilume-30 (Packard Instru- o., Downers Grove, IL) was added to il. Counts were performed in a Pack- quid Scintillation Counter (Model

tion of interferon and interferon as- ouse spleen cell cultures were pre- xactly as described for the *in vitro* sponse except SRBC were omitted. .5 μg/ml) was added to cultures for ı induction of interferon. Cultures ubated for 48 hr under the conditions d for the *in vitro* PFC response (13). supernatants were harvested by cen- ion at (400*g* for 10 min). Supernatants her assayed for interferon activity on of harvest or frozen immediately and t −70° for no more than 5 days before . Interferon activity was quantitated ıtiter plates by the method of plaque ın of vesicular stomatitis virus (40 ıallenge dose) in mouse L cells. Meth- ıse was used as an overlay (16) rather boxymethyl-cellulose.

ent cell cultures. Peritoneal exudate re obtained by injecting 8 ml of 37° medium intraperitoneally, then with- the injected fluid. The exudate cells ashed by centrifugation and resus- in culture medium. Sufficient cells ded to culture dishes so that approx- 2×10^5 adherent cells per dish (17) ıtained. Adherent cell cultures were three times gently with culture me- efore GA-treated spleen cells were

ts and discussion. Table I presents ım parallel experiments on the indi- and combined effects of GA, SEA, ME on the direct anti-SRBC PFC e, interferon induction, and DNA s in mouse spleen cell cultures. SEA, an established T-cell mitogen and immune interferon inducer (9, 18), inhibited the PFC response by 90%. Previous studies have shown that this inhibition occurs via suppressor cell activation (9, 11, 19). As shown, the inhibition of the PFC response was associated with induction of immune interferon and stimulation of DNA synthesis (as reflected) by increased ^{3}H-thymidine incorporation into DNA in the cells). GA alone blocked the normal PFC response and inhibited DNA synthesis. These effects were previously shown to be due to blockade of macrophage function (4, 5). GA blocked both SEA-induction of interferon and stimulation of DNA synthesis.

Although 2-ME (5×10^{-5} *M*) alone stimulated DNA synthesis and slightly enhanced the PFC response, it did not induce interferon. This is consistent with its property of replacing some macrophage function(s) (20, 21). Neither the PFC suppressor cell effect of SEA nor SEA-induction of interferon and stimulation of DNA synthesis were blocked by 2-ME. In fact, 2-ME enhanced the interferon-inducing property of SEA, which is known to require macrophage help. Both the PFC suppressive effect and the DNA synthesis inhibitory effect of GA were completely blocked by 2-ME. This is presumably due to 2-ME replacing a blocked macrophage function. The inhibitory effect of GA on SEA-induction of interferon and stimulation of DNA synthesis was blocked by 2-ME, which is able to substitute for macrophage function(s) (20, 21). Thus, as expected, the substitution by 2-ME for the macrophage function(s) inhibited by GA, permitted SEA to suppress the PFC response. Reversal by 2-ME of the interferon lymphokine inhibitory effect of GA indicates that another macrophage-dependent function is blocked by GA.

This blockade of macrophage function by GA, as measured by decreased interferon activity, was further investigated by reconstitution with untreated macrophages. Specifically, 1.5×10^7 spleen cells per ml in culture medium were incubated for 24 hr with 20 μg GA per ml. Excess GA was removed from spleen cells by washing twice with minimal essential medium, followed by the addition of the treated cells to adherent peritoneal cells from mouse peritoneal exudate (17). SEA, 0.5 μg/ml, was added to the cultures and inter-

TABLE I. EFFECT OF GALLIC ACID (GA) ON THE MOUSE SPLEEN CELL ANTI-SRBC PFC RESPONSE, AND SEA INDUCTION OF IMMUNE INTERFERON AND DNA SYNTHESIS.

Treatment	Direct anti-SRBC PFC/culture (Mean ± SEM)	Units interferon/ml (Log_{10})	^{3}H-thymidine uptake, cpm 10^{-2} (Mean ± SEM)
None	3850 + 29[a]	<0.5[b]	187 ± 13[c]
SEA (0.2 μg/ml)	383 ± 192	1.85	814 ± 70
GA (10 μg/ml)	<50	<0.5	98 ± 5
GA + SEA	<50	<0.5	90 ± 7
2-ME[d]	4917 ± 770	<0.5	306 ± 15
SEA + 2-ME	150 ± 150	2.45	683 ± 42
GA + 2-ME	5217 ± 235	<0.5	275 ± 9
GA + SEA + 2-ME	425 ± 25	2.25	507 ± 19

[a] Mean ± SEM of triplicate determinations, background corrected; data are representative of several experiments.
[b] Mean of duplicate determinations for each dilution; data are representative of at least two experiments.
[c] Mean ± SEM of triplicate determinations; data are representative of several experiments.
[d] Added to final concentration of 5×10^{-5} *M*.

feron concentrations were determined after 48-hr incubation (9). The adherent cells restored the ability of SEA to induce immune interferon in GA-treated cultures (compare line 5 with line 4, Table II). The combined interferon output of the GA-treated spleen cell control and untreated adherent cell control was only one-fourth that of the macrophage-reconstituted cultures. This pattern of interferon induction was shown in repeated experiments. Cultures that did not receive SEA contained less than 0.5 units of interferon per ml. The data are consistent with previous studies showing that macrophages are required for optimal production of immune interferon in humans (12).

An alternate interpretation of the above findings is that GA inactivated the induced interferon rather than blocked its induction. To test this possibility, immune interferon was incubated with various concentrations of GA at 37° for 1 hr and titrated for residual antiviral activity (Table III). At concentrations as high as 10 μg per ml, GA had no inhibitory effect on already induced interferon. Thus, GA blocked the induction of immune interferon but did not block the establishment of antiviral activity of already induced interferon.

The interactions of immunoregulatory cells are poorly understood; however, loss of suppressor cell function in the gut could contribute to autoimmune disorders (22, 23), altered mucosal penetrability, and increased IgE immunoglobulin formation (25). The data show that GA is capable of blocking a macrophage-dependent T-cell function (induction of immune interferon) that may be associated with suppressor cell activity. Since GA and related metabolites occur in highest concentration in the gastrointestinal tract and liver, their effects should be greatest in these organs.

TABLE II. SEA INDUCTION OF INTERFERON IN MOUSE SPLEEN CELLS; ADHERENT CELL REVERSAL OF GA SUPPRESSION.

Spleen cells	Treatment: SEA	Treatment: GA[a]	Treatment: Adherent cells[b]	Units interferon per ml (Log_{10} ± SEM)
+	+	−	−	3.00 ± 0.00 (1000)[c]
+	+	−	+	2.63 ± 0.18 (427)
−	+	−	+	1.43 ± 0.18 (27)
+	+	+	−	1.87 ± 0.09 (74)
+	+	+	+	2.63 ± 0.32 (427)

[a] Cultures incubated 24 hr with 20 μg/ml GA, then washed and restored in culture medium.
[b] Approximately 2×10^5 adherent mouse peritoneal cells.
[c] Concentrations of interferon expressed numerically; data are presented as the mean ± SEM of duplicate determinations; patterns of interferon production are representative of at least two experiments.

TABLE III. EFFECT OF GA ON THE ESTABLISHED ANTIVIRAL ACTIVITY OF SEA-INDUCED IMMUNE INTERFERON.

GA[a] (μg per ml)	Residual interferon activity (Log_{10} mean ± SEM)
0	2.45 ± 0.05
2	2.40 ± 0.00
5	2.35 ± 0.15
10	2.45 ± 0.05

[a] GA and SEA-induced interferon were incubated together at 37° for 1 hr and the residual antiviral activity was determined as previously described (9). Data presented as mean ± SEM of duplicate determinations; replicate experiments showed GA had no effect on established antiviral activity of immune interferon.

We have previously shown and have further demonstrated here that GA is capable of blocking a macrophage-dependent helper cell function (PFC response to SRBC) (4, 5). GA provides a chemical probe for specifically blocking at least two macrophage-dependent T-cell functions. Another food additive, carageenan, affects various macrophage-dependent immunologic events *in vitro* (26, 27) and depresses resistance to virus-induced tumors in mice (28). Because GA is a normal and additive-type dietary component consumed in large amounts in various countries, this and related phenols and polyphenols should be further studied to determine possible biological consequences of ingestion.

Summary. Gallic acid (GA), a food constituent and food additive metabolite, blocked macrophage-dependent mitogen-induced interferon production and suppressor T-lymphocyte function. Interferon production was restored by adherent peritoneal macrophages or 2-mercaptoethanol, a macrophage substitute. GA failed to block the antiviral activity of preformed mitogen-induced interferon. Possible consequences of loss of lymphokine production and suppressor T-lymphocyte function(s) are discussed.

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Unscheduled DNA Synthesis in Rat Lens Epithelial Cells Induced by Alkylating Agents[1] (40123)

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Alkylating agents have been used frequently as tools to produce cataracts in experimental animals (1). Alkylating agents are known to interfere with normal cell division in lens epithelial cells (2), but the mechanisms by which the cataracts are produced remain obscure. Such compounds are well known for producing damage to DNA of other cell types which results in DNA repair. Recently we have shown that DNA repair synthesis is active in the lens cells following damage by uv light (3). The present experiments were done to determine whether alkylating agents could also induce such unscheduled DNA synthesis (DNA repair synthesis) (4) in lens epithelial cells. The ability to induce repair synthesis would demonstrate that alkylating agents interacted with and presumably damaged the lens epithelial cell DNA.

Materials and methods. Whole lenses from male Osborne-Mendel rats (175–200 g) were incubated in 4 ml of medium (5) containing nitrogen mustard (HN_2, 10^{-4}, 4-nitroquinoline-1-oxide (4-NQO, 2×10^{-5} *M*), or no drug. The HN_2 was made up directly in the medium. 4-NQO was made up as a 10^{-3} *M* solution in 30% ethanol and an aliquot of 80λ was added to 4 ml of the medium. The control lens received 80λ of 30% ethanol. After one-half hour, the lenses were placed in fresh medium containing 5 μCi/ml of tritiated thymidine (^{3}HTdR, 40–60 Ci/m*M*, New England Nuclear) and incubation continued for 2 hr. After that time, the lenses were rinsed three times in phosphate buffer, and placed in Carnoy's fixative. Whole mounts were prepared from lens epithelium (6), and were dipped in Kodak NTB-2 emulsion. The slides were developed for 2 weeks under refrigeration, developed with Kodak D-19, fixed with Rapid Fix, and stained with hematoxylin and eosin.

Results. Figure 1a demonstrates the typical nuclear labeling pattern which was observed following incubation of a rat lens with NH_2 followed by ^{3}HTdR. This type of diffuse nuclear labeling is typical of DNA repair synthesis such as has previously been observed in HeLa (7) and lens cells (3). This type of labeling was not observed in control lenses (Fig. 1b) which were not subjected to the alkylating agent. The heavily labeled cells which are seen in both figures are typical of replicative DNA synthesis (8), which can be seen to persist in the NH_2 treated lenses.

A diffuse labeling pattern consistent with DNA repair synthesis was also induced by exposure of the lens to 2×10^{-5} M-NQO. This alkylating agent also did not interfere with replicative type labeling at the concentration used.

Discussion. Many alkylating agents, including HN_2 (1), have been shown to cause cataracts. The opacities are generally posterior subcapsular cataracts which do not appear for months or years after the initial exposure. Such similarities to ionizing radiation effects have caused alkylating agents to be referred to as "radiomimetic" (1). Evidence is strong that the primary site of action of X-rays in the lens is to the epithelial cells rather than to the cortical fibers already formed (9). Our results show that the lens cell DNA is also one target of alkylating agents since unscheduled DNA synthesis in the epithelial cells followed exposure to the drugs. In other cell systems, DNA damage which is unrepaired or "misrepaired" (10) has been proposed to cause cellular aging (11) and mutations (12). One might expect these agents to produce similar effects in the lens cells.

The results reported here support the suggestion that certain cataractogenic agents have their initial effect on the lens epithelial

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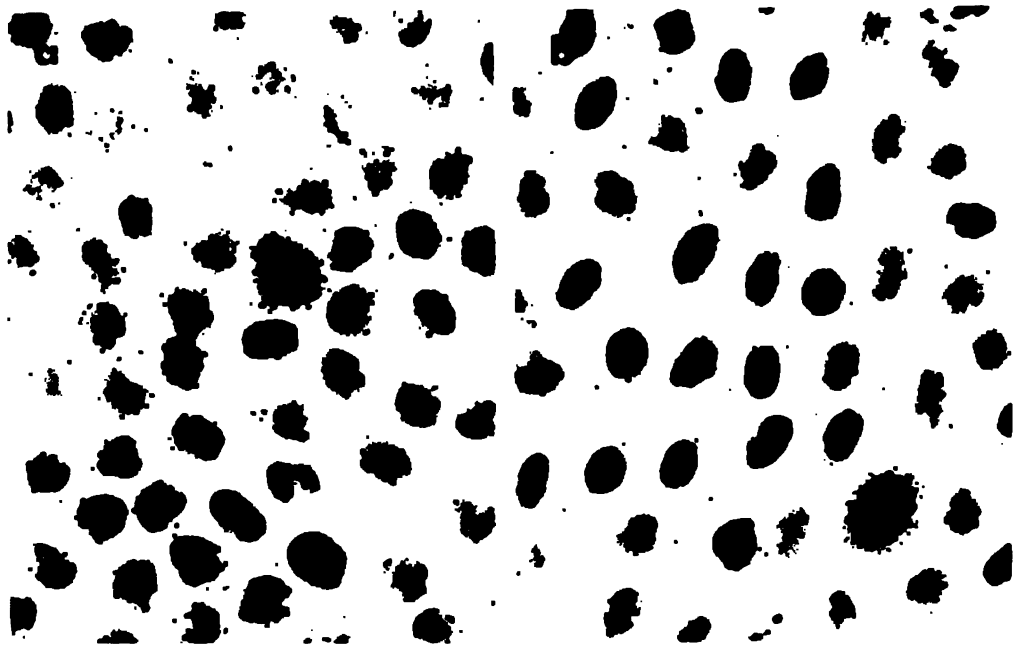

FIG. 1. Autoradiograms of epithelia prepared from whole rat lenses incubated *in vitro* with 3 HTdR (a) following one half hour's incubation with 10^{-4} *M* HN_2 (b) untreated control.

cell DNA. If this initial damage is not repaired, or is misrepaired, the cell may express itself abnormally in terms of cell division, protein synthesis, and differentiation into fibers with ultimate opacification. These events may occur with considerable time delay due to the normal differentiation scheme for lens epithelium.

The importance of the repair process for maintenance of normal lens clarity is suggested by split dose ionizing radiation studies. A single dose of X-ray which produces opacification fails to produce cataracts if the total dose is split and given in several small increments. In the case of neutron irradiation, splitting a cataractogenic dose does not decrease the severity of the cataract produced (13). In contrast to X-rays, which produce repairable single strand breaks, neutrons produce a high proportion of nonrepairable double strand breaks (14). Thus, splitting an X-ray dose allows time for the smaller amounts of damage to be repaired before additional damage is imposed by subsequent doses. No benefit results from splitting the neutron dose since little repair occurs in the interim.

Summary. Two alkylating agents, HN_2 and 4-NQO were found to induce repair synthesis of DNA in rat lens epithelial cells. HN_2 is known to produce cataracts similar to radiation cataracts. A mechanism by which radiomimetics may produce cataracts is proposed.

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Serum Triiodothyronine Concentrations in Riboflavin-Deficient, Diabetic and Normal Mice (40124)

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Certain investigations have demonstrated a direct relationship between thyroid hormone and riboflavin metabolism (1, 2). Hepatic concentrations of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and free riboflavin are significantly decreased in hypothyroid and increased in hyperthyroid rats (1). Similarly, hepatic activity of flavokinase, which converts riboflavin to FMN, is decreased in hypothyroid animals (1). Administration of thyroid hormone produces nearly a twofold increase in enzyme activity of these animals (1). A similar hepatic profile as in hypothyroidism with regard to concentrations of flavin coenzymes and flavokinase activity was demonstrated in riboflavin-deficient rats (1). Consequently, a biochemical similarity between hypothyroidism and riboflavin deficiency was proposed (3).

An additional interrelationship between thyroid hormone and riboflavin metabolism was described by Galton and Ingbar (4). These authors reported a significantly decreased hepatic deiodination of thyroxine in riboflavin-deficient rats. The deiodination of thyroxine was not affected in the kidney, muscle or brain. In addition, these authors reported that the urinary excretion of ^{131}I and the serum concentration of protein-bound iodine were similar in normal and riboflavin deficient animals. By contrast, Nolte *et al.* (5) reported a significant decrease in serum immunoreactive thyroxine concentration in riboflavin deficient rats. Since spontaneous riboflavin deficiency was reported to occur in genetically diabetic KK mice (6), the present study was undertaken to investigate whether or not the serum triiodothyronine concentrations are altered in these mice.

Material and methods. The Swiss albino (SA) and KK (genetic diabetic) mice have been inbred in our laboratory for several generations. They were fed the Old Guilford mouse laboratory chow (The Emory Morse Co., Guilford, CN) *ad libitum*. Both groups of mice were maintained under similar laboratory conditions. The animals in this part of the study were males between 8 and 9 months of age, and were tested for riboflavin nutritional status.

Riboflavin deficiency was produced in 40-day-old male SA mice. They were purchased from Camm Research Institute, Wayne, NJ. The mice were divided into two groups. One group was placed on a riboflavin deficient diet (ICN Life Sciences Group, Cleveland, OH) for 50 days. The other group was fed the Old Guilford mouse laboratory chow for a similar period of time. Both groups of mice were fed the respective diets *ad libitum*. The mice on riboflavin deficient diet lost weight and showed signs of the deficiency.

All groups of mice were maintained in plastic cages with sanichips as bedding. Six KK mice were given 140 μg of riboflavin per mouse per day s.c. in 0.9% saline for 20 days and then tested for riboflavin nutritional status.

Prior to the study, the mice were fasted for 18 hr with free access to water. Blood was drawn from the orbital plexus. Serum was collected by centrifugation of blood at 10,000g for 10 min. All serum samples were stored at $-25°$ until analyzed for T_3 concentrations.

The riboflavin nutritional status of each animal was assessed by determining the glutathione reductase activity in erythrocyte hemolysates (7). The hemolysates were prepared as follows: about 0.3–0.35 ml of whole blood was transferred into tubes containing 0.1 ml of ACD solution (7.3 g of anhydrous citric acid, 22.0 g of sodium citrate and 24.5 g of glucose made up to 1 liter with water) and centrifuged at 200g in a refrigerated centrifuge for 10 min. The plasma and buffy coats were removed with a Pasteur pipette, and the red cells were mixed with 1.0 ml of

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ıdium chloride. The suspension was ged at 200*g* for 10 min, and the su- nt solution was removed and dis- The red cells were mixed again with nd centrifuged as before. In this way, cells were washed 3 times. To prepare ıolysate, 0.1 ml of erythrocyte suspen- ıs diluted to 2.0 ml with cold distilled nd allowed to stand at 4° for 45 min. ıpension was centrifuged at 800*g* for in a refrigerated centrifuge and the ıtant solution was separated and ıt −25° until assayed.

ıthione reductase activity in the hem- was determined by the method of ın and Sauberlich (7). The assay me- ontained 2.0 ml of 100 mM potas- ıdium phosphate buffer, pH 7.4, 0.1 emolysate, 0.1 ml of 7.4 m*M* oxidized ione (GSSG), 0.05 ml of 80 m*M* Na_2, 0.1 ml of 1.9 m*M* NADPH, 0.1 .27 m*M* FAD and distilled water to a ılume of 2.5 ml. Two tubes were used ı assay, one containing FAD and the ıithout FAD. Blanks for these tubes ed the respective reactants except sates. All the reactants except H were mixed thoroughly and incu- ıt 37° in a water bath for 8 min. ing the preincubation, the reaction was by the addition of NADPH, and the ıbsorbance was measured at 340 nm eckman DB spectrophotometer. The were used to zero the instrument. The ı mixture was transferred into a tube ıubated again at 37° for 10 min. Fol- this incubation period, the reduction absorbance (oxidation) of NADPH asured. The results were expressed as coefficients (AC), calculated by di- he amount of reduction of absorbance DPH in the presence of FAD for 10 the amount of reduction of absorb- NADPH without added FAD for 10 C values from 0.9 to 1.3 were consid- ırmal, and those greater than 1.3 were d as evidence of riboflavin deficiency ed upon AC values, both SA and KK ere classified as normal (non-deficient) flavin-deficient.

ıdothyronine (T_3) concentrations were ined in 0.1 ml of serum by a radioim- ssay, with an RIA-Mat circulating ^{125}I–T_3 kit. The method is specific for measuring total circulating serum T_3 levels, because of the use of antibodies which react *in vitro* with T_3 but not with thyroxine or mono- and diiodotyrosines. Duplicate determinations were performed only in 13 samples, since sufficient amount of serum was not available in the remaining samples. The difference between the duplicate samples varied from 3 to 7.5%. In order to determine the reproducibility of the assay, the T_3 levels were determined in five samples 20 days after the original determination. The standards and the reagents were used from the same kit. Five to ten percent decrease in the T_3 levels was found. The method is, therefore, accurate and reproducible in our hands. The T_3 levels were expressed as ng per 100 ml of serum. Determinations of T_3 levels were done in a blind fashion.

Student's *t* test for unpaired samples was applied to calculate the significance of the difference between means of observations. *P* values greater than 0.05 were considered nonsignifcant.

Chemicals. GSSG, NADPH, FAD and riboflavin were obtained from Sigma Chemical Co., St. Louis, MO, and ^{125}I–T_3 kit including standards from Mallinckrodt-Nuclear, St. Louis, MO.

Results. Table I shows the mean AC values and serum T_3 levels in aged SA and KK mice. The AC values of riboflavin-deficient SA and KK mice did not differ significantly from each other. When six deficient KK mice were supplemented with riboflavin, their AC values returned to normal. There was no significant difference in T_3 concentrations between normal and riboflavin-deficient SA mice. Also no significant difference in the T_3 level was found between normal KK and deficient KK mice. Furthermore, administration of riboflavin to deficient KK mice did not alter the T_3 concentrations. No statistically significant differences in T_3 levels were found between normal SA and normal KK or between riboflavin-deficient SA and riboflavin-deficient KK mice.

Induced riboflavin deficiency in young SA mice also caused no difference in the serum T_3 concentration (Table II). Although the mean T_3 level was 30% higher in riboflavin-deficient mice, the increase was not statisti-

TABLE I.[a]

	SA		KK		Deficient KK + Riboflavin
	Normal	Deficient	Normal	Deficient	
AC	1.24 ± 0.02 (8)	1.60 ± 0.06 (5)	1.00 ± 0.02 (3)	2.01 ± 0.40 (5)	1.15 ± 0.03 (6)
T_3	49.00 ± 6.00 (8)	58.00 ± 5.00 (5)	77.00 ± 17.00 (3)	69.00 ± 13.00 (5)	49.00 ± 9.00 (6)

Significance (T_3)
Normal SA vs. Deficient SA : NS
Normal KK vs. Deficient KK : NS
Normal KK vs. Deficient KK + riboflavin : NS
Normal SA vs. Normal KK : NS

[a] Activity coefficients (AC) for glutathione reductase activity in erythrocyte hemolysates and serum triiodothyronine (T_3) concentrations (ng/100 ml) in Swiss albino (SA) and genetically diabetic (KK) mice aged 8–9 months. Values shown are means ± SE. Numbers in parentheses indicate number of animals.

TABLE II.[a]

Normal	Riboflavin-deficient
73.00 ± 15.00 (7)	95.00 ± 12.00 (6)[b]

[a] Serum triiodothyronine levels (ng/100 ml) in Swiss albino mice fed control and riboflavin deficient diets for 50 days. Each value is the mean ± SE. Numbers in parentheses represent number of animals.
[b] Normal vs riboflavin deficient : p = NS.

cally significant because of the variability of the data.

There was no difference in the fasting blood sugar levels between normal and spontaneously developed riboflavin-deficient SA or KK mice. However, in mice with induced riboflavin deficiency a significant decrease ($P < 0.001$) in the fasting blood sugars was found when compared to mice fed a normal diet *ad libitum* or pair-fed to the riboflavin-deficient group.

Discussion. It has been shown that determination of the erythrocyte glutathione reductase, an FAD-containing enzyme, is an accurate procedure for assessing the riboflavin nutritional status in rats (7) and humans (9–11). In the deficient state, enzyme activity is stimulated *in vitro* by FAD (12, 7) and *in vivo* by riboflavin intake (13). The extent of stimulation of the erythrocyte glutathione reductase *in vitro* is inversely related to the FAD saturation of the enzyme protein, which in turn is dependent on the availability of riboflavin. Glatzle *et al.* (10) have reported the percentage of stimulation of the NADPH-dependent erythrocyte glutathione reductase in the presence of added FAD as a measurement of the riboflavin status in man. They referred to this stimulatory effect as activity coefficient (AC). We employed the erythrocyte glutathione reductase assay system to assess the riboflavin nutritional status of aged normal control SA and genetically diabetic KK mice.

The KK mice are an inbred strain of animals with spontaneous hereditary diabetes. They develop impaired tolerance to oral glucose (after an 8 hr fast) between 2 and 3 mon of age. This impairment becomes more pronounced at 9 mon of age. Since these mice demonstrate mainly impaired tolerance to glucose with normal fasting blood sugars, they are classified as chemical diabetics (14).

The present report demonstrates that spontaneous riboflavin deficiency occurs in aged SA and KK mice on a chow diet. This deficiency was not due to a low caloric or riboflavin intake, because these mice consumed a regular quantity of food (the intake of food was measured) which had the required amount of riboflavin (3 mg/lb). This deficiency has been found to be more common among KK than among SA mice (6). Administration of riboflavin to deficient KK mice returned the AC values to normal. A biochemical similarity between hypothyroidism and riboflavin deficiency has been demonstrated in that flavin coenzymes are decreased in the liver in both conditions (3). In view of this similarity, it is of interest that serum T_3 levels remain normal in mice with spontaneous or induced riboflavin deficiency.

Galton and Ingbar (4) found no difference in serum protein-bound iodine levels between normal and riboflavin-deficient rats. On the other hand, Nolte *et al.* (5) reported significantly decreased levels of serum thyroxine in riboflavin-deficient rats. Nevertheless, and in spite of the previous observation that deiodination is impaired in riboflavin deficiency (4), the serum T_3 levels were unaltered in

avin-deficient mice. Furthermore, sup-
:ntation of riboflavin to deficient KK
did not alter the T_3 levels. Additional
:s including measurements of thyroxine
are indicated. The failure to observe
;es in circulating T_3 levels in riboflavin
ency, associated either with genetic di-
s or dietary restriction, suggests that thy-
ùnction remains normal in this condi-
This is in spite of the fact that hypothy-
m and riboflavin deficiency have simi-
fects on hepatic flavin coenzyme levels

nmary. The riboflavin nutritional status
ssessed by activity coefficient (AC) of
hione reductase in erythrocyte hemo-
s of normal Swiss albino (SA) and ge-
ılly diabetic (KK) mice aged 8–9
ıs. AC values greater than 1.3 were
lered as evidence of riboflavin defi-
/. Based upon AC values, both SA and
ıice were divided into normal and ribo-
-deficient groups. In some young SA
riboflavin deficiency was produced by
ıg of a riboflavin-deficient diet. In both
al and riboflavin-deficient mice, the se-
L-triiodothyronine (T_3) concentrations
determined by radioimmunoassay. The
s indicate that the T_3 concentration is
fected either in riboflavin deficiency or
ıetic diabetes.

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Malignant Conversion of Shope Papillomas and Associated Changes of Serum Ceruloplasmin in Domestic Rabbits (40125)

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Shope papillomas induced in domestic rabbits are converted into squamous cell carcinomas when allowed to grow on skin of the animals for 12 months or more (1). As reported in our previous paper, the papillomas induced in newborn domestic rabbits grew more than 6 months in the majority of animals without sign of regression (2). The malignant conversion began to take place in most of such papillomas after the lapse of 1 year, concomitantly with blueing of the serum in these animals. Bluish serum has also been observed in rabbits bearing Vx2 and Vx7 carcinomas (3). The bluish color of the serum is ascribed to the augmented level of serum ceruloplasmin, a copper-containing blue enzyme. The present paper reports the quantitative and qualitative changes of serum ceruloplasmin, associated with the malignant conversion of Shope papillomas to carcinomas.

Materials and methods. Rabbits. The papillomas were induced in neonatal rabbits with intradermal injection of Shope papilloma virus (SPV). These animals were from the same group as used in the previous report (2). In addition, some domestic rabbits were used for induction of the papillomas, transplantation of Vx2 carinomas, and for mating. Sera obtained from these animals were kept frozen at −20° until use. Fresh sera were also subjected to comparative analysis.

Histological examination. Biopsy specimens were taken from the tumorous tissues periodically and were diagnosed according to the pathological criteria for malignancy.

Quantitative estimation of serum copper level. Concentration of serum copper was measured according to the Bathocuproine method (4) and also by the atomic absorption spectrophotometry (5).

Disc electrophoresis and stainings. Disc electrophoresis of serum was performed in 5% polyacrylamide gel under the conditions described by Ornstein (6). The protein was stained with Amido black 10B and the enzymatic activity was manifested by soaking the gel overnight at 37° in 0.05% *p*-phenylenediamine in 0.1 *M* acetate buffer pH 5.7.

Immunoelectrophoresis. Immunoelectrophoretic analysis was performed in 1.2% agarose gel according to the micromethod (7). Antisera for human ceruloplasmin and for whole rabbit serum were purchased from Hyland (Costa Messa, USA) and Biken (Osaka, Japan), respectively.

Results. Serum copper level in rabbits which encountered malignant conversion of the papillomas. Serum copper level was assayed and comparison was made among the sera of seven tumor-bearing rabbits at 4 months of age and later just before their death due to the malignancy. In these animals, ulceration of papillomas was observed around 12 months after the tumor induction and was followed by the swelling of regional lymph nodes. At autopsy, lung metastasis was observed in all cases. Malignant conversion was confirmed by histological examination of ulcerated portions of the tumor in all animals. As shown in Table I, serum copper level before death was elevated 2–6 times as compared to that at 4 months of age.

Disc electrophoretic pattern of ceruloplasmin activity. A conspicuous single band of enzymatic activity was manifested in the serum at 4 months of age, whereas a faint second band with a larger mobility appeared in the serum from the same animal when ulceration of papilloma was first noticed, and such double band became apparent 4 months later when ulceration was exacerbated (Fig. 1A). Similar findings were observed in the sera from all seven animals examined. The double bands were also observed in rabbits transplanted with Vx2 carcinomas (Fig. 1B). A single band in the normal serum and the double bands in the carcinomatous serum were manifested, irrespective of fresh or frozen serum.

Changes in serum copper level, disc electrophoretic and immunoelectrophoretic pattern of ceruloplasmin in a single rabbit, associated

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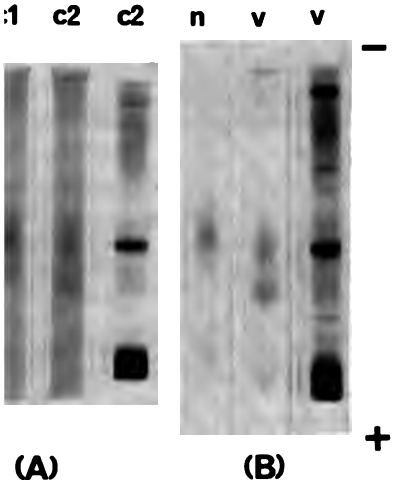
. SERUM COPPER LEVEL IN RABBITS WITH JS-INDUCED PAPILLOMAS AND AT THE MALIGNANT CONVERSION.

Serum copper[a] (μg/dl)		Occurrance (months) of	
At 4 months	Before death	Ulceration	Death
91	358	15	17
131	353	21	25
108	324	17	18
97	324	16	18
125	301[b]	14	—[c]
74	438	12	14
108	319	13	16

nined by the Bathocuproine method.
red at 5 months after ulceration was noticed.
l surviving.



Disc electrophoretic pattern of ceruloplasmin malignant conversion (A) and at Vx2 trans- (B). (A), Sera from rabbit No. 215. p: 4 age, c1: 14 months of age when ulceration of was noticed, c2: 5 months thereafter. Three the left were stained for ceruloplasmin activ- e 4 for protein. (B), Sera from rabbit No. 322. v: 2 months after Vx2 transplantation. Two the left were stained for ceruloplasmin activ- e 3 for protein. Anode is downward.

nancy, malignant conversion and total of tumor. Serum copper level was be elevated approximately twice at ɔn and four times at malignant con- ıs compared with that of the normal ig. 2). The elevated level in carci- s serum dropped to the normal level dl at 1 month after total resection of ır. Ceruloplasmin activity was man- disc electrophoresis as a single band ıl serum and at parturition, and as ɔands in carcinomatous serum. Immunoelectrophoretic analysis revealed a single precipitation arc in sera from both pregnant and carcinomatous animals, but the arc formed in the latter showed more widely distributed pattern of the precipitate. These results were consistent with those obtained by disc electrophoretic analysis.

Discussion. Elevation of serum copper or ceruloplasmin level is known to occur in diseases such as cancer, inflammation, biliary duct obstruction (8–10). In cancer patients, elevated serum copper level is reportedly high at approximately twice the normal level. In the present work with rabbits, we found the augmentation of serum copper and a possible qualitative change of serum ceruloplasmin accompanying the malignant conversion of Shope papillomas. Ulceration is assumed to be the first macroscopic sign of benign-to-malignant conversion taking place in the tumor system (1). The bluish coloration of the serum, as reported herein, is also indicative of such malignant alteration. Although a direct relationship was not established, it is plausible to assume that the bluish color is due to the elevated level of serum ceruloplasmin, as indicated by elevated copper levels and intense ceruloplasmin activity in the serum specimens.

Physiological augmentation of ceruloplasmin occurs also in association with pregnancy (9). Comparison of such augmented ceruloplasmin was made in a single rabbit at pregnancy and at malignant conversion and a qualitative difference was evident among both sera. The enzymatic activity was manifested as a single band at pregnancy and as double bands at malignant conversion. One might argue that the double band formation could be due to artifacts occurring with storage and that loss of enzymatic activity may have taken place during storage for one component and not for the other. However, fresh sera from carcinomatous rabbit also gave double bands and fresh normal sera a single band, thus excluding such possibilities. Two distinct ceruloplasmin activities could be due to two distinct isozymes of ceruloplasmin, although in the present experiment, we could not exclude the possibility that one band is due to a degraded product or a complex of the native enzyme. The presence of ceruloplasm in isozymes has been reported in human serum (11) but not in rabbit serum. Our

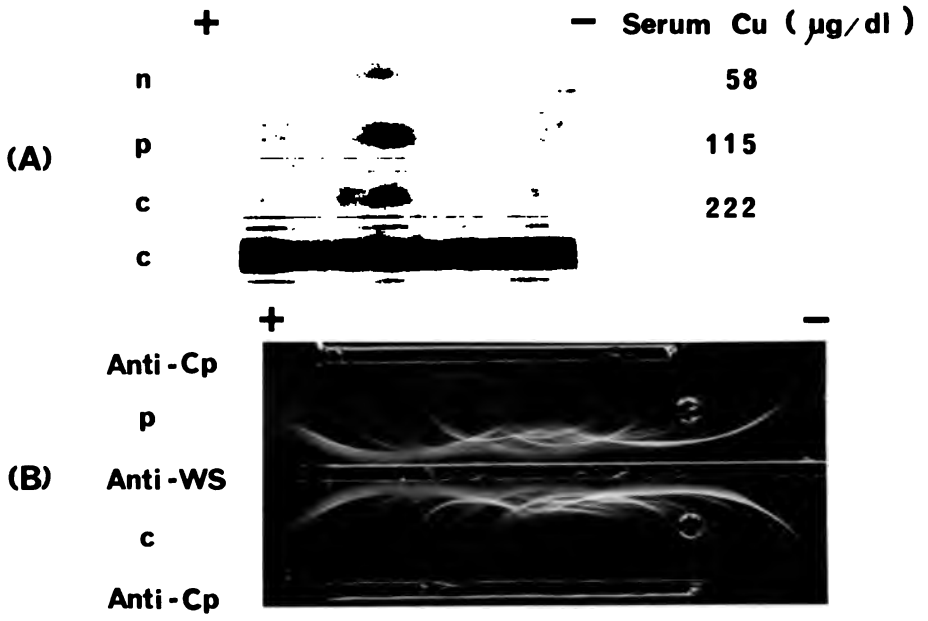


FIG. 2. Disc electrophoretic and immunoelectrophoretic patterns of ceruloplasmin in sera from a single rabbit No. 176 at parturition and at malignant conversion. (A), Disc electrophoretic pattern of enzymatic activity. n: normal, p: at parturition, c: at malignant conversion. Three tubes from the top were stained for enzymatic activity and tube 4 for protein. Each tube is annexed with serum copper level measured by the atomic absorption spectrophotometry. (B), Immunoelectrophoretic pattern of the same sera in (A). Anti-Cp: anti-human ceruloplasmin serum, Anti-WS: anti-rabbit whole serum. Anode is to the left.

preliminary results on heat-stability, optimal pH range and inhibition by NaN_3 have shown substantial differences between the enzymatic activities of the two components, and such finding is consistent with the assumption that these are indeed isozymes. An invariable symptom as such may be utilized as a marker for the malignant conversion of benign tumors.

Summary. Shope papillomas neonatally induced in domestic rabbits were observed to convert into squamous cell carcinomas after the lapse of a year or more. The ulceration of the tumor, the bluish coloration of the serum and the elevation of serum copper level were concomitantly observed in these animals. The serum copper was found to be augmented two to six times higher among animals with malignancies than those with benign papillomas. Disc electrophoretic analysis of the serum showed that ceruloplasmin activity was manifested as single and double bands, before and after the malignant conversion, respectively. The serum at parturition also showed an elevated serum copper level but only a single band of ceruloplasmin appeared in disc electrophoretic analysis.

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Author Index for Volume 157

A

, R., 656
C., 407
G. D., 556
393
Q., 297
, 511
, W. A., 489
). L. 684
M., 189
A., 235
g, W. D., 363
A. E., 421

B

T., 1
H., 536
H., 139
T. A., 656
S. M., 648
f. H., 386
U. H., 348
P., 106
H. H., 12
', C. D., 5
, G. S., 461
., 506
J., 125
. M., 472
k, R., 354
S., 424
eault, G. A., 402
J. M., 148
L. L., 590
E. F., 220
l., 220
J. D., 517
. D., 211
ı, B., 449
R. W., 580
E. B., 636
W., 572

C

. H., 194
l., 148
er, G., 256
). H., 378
J., 97
., 211
.. Y., 319
., 643
L. M., 206
Choi, Y. C., 569
Cholvin, N. R., 610
Chopra, I. J., 106
Cian, F., 342
Coe, J. E., 289
Coffey, J. W., 435
Cole, B. C., 83
Collins, R., 75
Cook, J. D., 481
Coon, R. L., 36
Cooper, C. W., 374
Cooperman, J. M., 41
Cornell, L., 665
Craighead, J. E., 500
Crass, M. F., III, 486
Crouch, N. A., 225

D

Dabich, D., 175
Dalferes, E. R., 461
Davidson, W. L., 148
Davis, B. K., 54
Davis, D. L., 536
Davis, J. O., 116, 245
Davis, M., 70
Davis, S. L., 106
DeForrest, J. M., 245
Degré, M., 253
Deibler, G., 194
Del Soldato, P., 615
DeMeio, J. L., 79
de Nicola, G., 342
DeSanctis, A. N., 79
Desplan, C., 241
DeVore, V., 5
DiBona, G. F., 453
Dodge, W., 312
Doelger, S. G., 134
Dole, H. S., 41
Dore-Duffy, P., 622
Downey, S., 550
Ducayen, M., 550

E

Eade, M. N., 390
Eaton, J. W., 506
Ebarb, R. L., 262
Edstrom, R. D., 411
Efremov, G. D., 250
Eklund, D. L., 134
Ensinck, J. W., 89, 643
Entringer, M. A., 337
Ercoli, N., 397
Ershoff, B. H., 626
Eylar, O. R., 322

F

Fang, V. S., 159
Farmer, J. M., 256
Farquhar, J. D., 79
Fernandez-Durango, R., 235
Fiedler-Nagy, C., 435
Fine, L. G., 189
Fishback, J., 235
Fleming, W. W., 200
Fliedner, T. M., 301
Fontebuoni, A., 184
Forghani, B., 268
Forman, L. P., 418
Fraley, D. S., 393
Frank, L., 293
Freeman, R. H., 116, 245
Fregly, M. J., 18
Friedman, H., 381, 631
Frost, H., 61
Frost, P., 61
Fujimoto, W. Y., 89
Fuller, R. W., 202
Furuhashi, N., 159

G

Gaetjens, E., 517
Gala, R. R., 415
Gallagher, R. E., 312
Garry, R. F., 125
Gauderer, C. A., 129
Gazis, D., 584
Gebber, G. L., 648
Gennaro, R., 342
George, D. T., 1
George, W. J., 580
Germain, M. J., 262
Gibbs, G. E., 70
Gilmore, J. P., 70
Ginn, R. W., 390
Glasgow, L. A., 83
Gleich, G. J., 129
Goetz, K. L., 531
Goldberg, M. E., 121
Goldberg, M. R., 590
Goldstein, E. J., 189
Gomez, O., 159
Goonan, W., 550
Greenberg, B. P., 541
Gresser, I., 456
Grilli, G., 184
Gromisch, D. S., 41
Grossman, M. I., 440
Guntheroth, W. G., 610

0037-9727/1574-0697$01.00/0

H

Haldar, J., 476
Hankins, W. A., 79
Harter, J. M., 139
Hartman, A. D., 297
Harvey, J. E., 553
Hedges, A. R., 94
Hedges, K., 94
Hedlund, L., 134
Heintz, N., 500
Hemrick, S. K., 202
Herbert, D. C., 605
Herron, J., 75
Hill, P., 231
Hilleman, M. R., 148, 636
Hirt, H. M., 29
Hodson, C. A., 144
Hollingsworth, D. R., 106
Holloman, T. L., 256
Hornshuh, M. J., 278, 402
Howe, C., 622
Hsiung, G. D., 569
Huisman, T. H. J., 250

I

Igler, F. O., 36
Ishikawa, H., 605
Ito, Y., 694

J

Jacobs, J. J., 163
Jaffe, I. A., 155
Jagiello, G., 550
Jarboe, G. E., 57
Jeevanandam, M., 282
Johnson, D. E., 672
Johnson, D. G., 89
Johnson, H. D., 134
Johnson, H. M., 684
Johnson, S. M., 200
Jose, J. G., 688
Jullienne, A., 241

K

Kamo, I., 381
Kampine, J. P., 36
Kampschmidt, R. F., 669
Kaufmann, J., 75
Kawabori, I., 610
Kelleher, D. L., 18
Keller, N. R., 369
Kelman, B. J., 57
Kennedy, B. J., 411
Kiang, D. T., 411
Kim, Y. S., 466
Kinney, J. M., 282
Kirchner, F. R., 572
Kirchner, H., 29
Kitay, J. I., 103
Klaassen, C. D., 66
Klahr, S., 168
Knodell, R. G., 306
Koerbling, M., 301
Koopmans, H. S., 430
Kot, P. A., 677
Krause, B. R., 297
Kritchevsky, D., 231
Kumar, M., 421
Kumpati, J., 250
Kwanyuen, P., 626

L

Lambooy, J. P., 466
Lawrence, A. M., 407
Lennette, E. H., 268
Lerner, A. M., 442
Leveille, G. A., 278, 402, 528
Levin, J. I., 576
Lindamood, C., III, 200
Lipschitz, D. A., 481
Lischko, M. M., 134
Lombardi, P. S., 83
Lombardini, J. B., 486
Long, C. L., 282
Lopez, R., 41
Lucarelli, G., 184
Lyons, S. F., 631

M

MacArthur, S. A., 18
MacPherson, B. V., 500
Madsen, D. C., 386
Makhlouf, G. M., 565
Malvin, R. L., 665
Mangoff, S. C., 110
Markham, P. D., 312
Martenson, R. E., 194
Martin, L. G., 681
Maryanski, J. L., 556
Mashburn, H., 75
Mattaliano, V. J., 435
McGill, H. C., Jr., 672
McGrath, J. J., 681
McLean, A. A., 148, 636
McMurray, D. N., 215
Medearis, D. N., Jr., 523
Meites, J., 144
Meli, A., 615
Mennes, P. A., 168
Merchant, F. W., 89
Meredith, R. F., 449
Mergenhagen, S. E., 29
Merriman, C. R., 669
Merryman, P., 155
Metzgar, D. P., 79
Milgrom, F., 511
Milhaud, G., 241
Miller, A. E., 494
Miller, E. R., 528
Miller, G., 489
Milner, J. A., 110
Moore, R. N., 125
Morris, R. E., 330
Morris, R. W., 194
Mossman, B. T., 500
Moukhtar, M. S., 241
Mueting, N. J., 531
Murphy, W. H., 330, 556
Murthy, V. S., 121
Murty, C. N., 660

N

Nance, S. L., 33
Nelson, D., 506
Nothdurft, W., 301
Nowotny, A., 348

O

Obie, J. F., 374
Ogle, T. F., 103
Okunewick, J. P., 449
Olson, L. S., 599
Ophaug, R. H., 363
Osborne, J. W., 572
Oster, J. R., 97
Overall, J. C., Jr., 83
Owens, M. S., 175

P

Paolini, N. S., 262
Parks, N. M., 23
Patterson, W. R., 273
Pauly, J. L., 262
Perez, G. O., 97
Persidsky, M. D., 599
Peterson, W. D., Jr., 326
Pollock, A. S., 481
Porcellini, A., 184
Porte, D., Jr., 643
Posner, A. S., 590
Prokay, S. L., 523

R

Race, R. E., 289
Radhakrishnamurthy, B., 461
Ramp, W. K., 358
Ramwell, P. W., 677
Raulais, D., 241
Rawls, W. E., 273
Reddi, A. S., 690

B. S., 94
I. C., 106
s, E. G., 605
M. A., 215
, C. A., 337
, W. B., 180
y, S. H., 665
G. D., 494
:, P., 241
s, R. J., 293
on, W. A., 337
W. R., 672
, D., 342
s, D. R., 278, 402, 528
. C., 677
ireich, D. L., 29
V. M., 301
ryn, L., 189
aist, O., 506

S

. I., 565
ddin, S. Z., 312
D. S. R., 660
L. B., 57
, W. H., 476, 584
, A. V., 235
lin, M., 565
ff, R. D., 354
ert, P. M., 472
t, N. J., 268
man, B. O., 418
ler, C., 342
tz, G. N., 424
tz, M., 256
. K., 326
., 694
. S. Y., 378
n, I. L., 50
M., 605
T. C., 326
ky, H., 660
, J. W., 402
ns, J. W., 144
L., 363
M., 23
Smith, J., 61
Smith, J. C., Jr., 211
Smith, K. O., 273
Smith, P. H., 89, 643
Smith, R. T., 354
Smith, S. T., 326
Smith, W. D., 12
Solomon, S., 12
Sonneborn, R. E., 97
Snoddy, H. D., 202
Srebnik, H. H., 553
Starcke, E. N., 50
Stephens, G. A., 116, 245
Stevenson, J. G., 610
Stringer, G. I., 44
Strunz, U. T., 440
Studzinski, G. P., 206
Stulberg, C. S., 326
Subramanian, M. G., 415
Sullivan, A. C., 435
Sutton, E. T., 536
Swack, N. S., 569

T

Tanapat, P., 517
Targowski, S. P., 511
Tepper, S. A., 231
Thomas, M. L., 358
Thomson, J. F., 33
Thrasher, T. N., 18
Tobin, R. B., 5
Toft, D. O., 594
Tokuda, H., 694
Tollaksen, S. L., 33
Tollerton, A. J., 134
Tomasi, T. B., Jr., 594
Trizna, W., 189
Trodahl, J. N., 50
Turner, R., 75

U

Upchurch, H. F., 669
Urnovitz, H. B., 556

V

Vaamonde, C. A., 97
Vella, P. P., 148
Verney, E., 660
Vignaux, F., 456
Volterra, G., 615
Voogt, J. L., 576

W

Waite, M. R. F., 125
Waldron, T. L., 121
Walsh, J. H., 440
Walter, B. K., 57
Wang, B. C., 531
Watkins, B. E., 116, 245
Watson, D. W., 472
Watson, R. R., 215
Weaver, E. V., 449
Webster, P. D., III, 23
Weibel, R. E., 636
Weir, W. C., 418
Wexler, B. C., 541
White, J. G., 506
Whitford, G. M., 44
Wicks, T. C., 677
Wilbur, R. L., 672
Wildman, S. G., 626
Williams, R. H., 89
Wilson, F. M., 442
Wisseman, C. L., Jr., 322
Woodhour, A. F., 148
Woods, S. C., 643
Wostmann, B. S., 386
Wurdeman, R., 5

Y

Yam, J., 293
Yates, J., 168
Yielding, K. L., 688
Yoshida, A., 369
Yu, S. Y., 369

Z

Zfass, A. M., 565
Zuperku, E. J., 36

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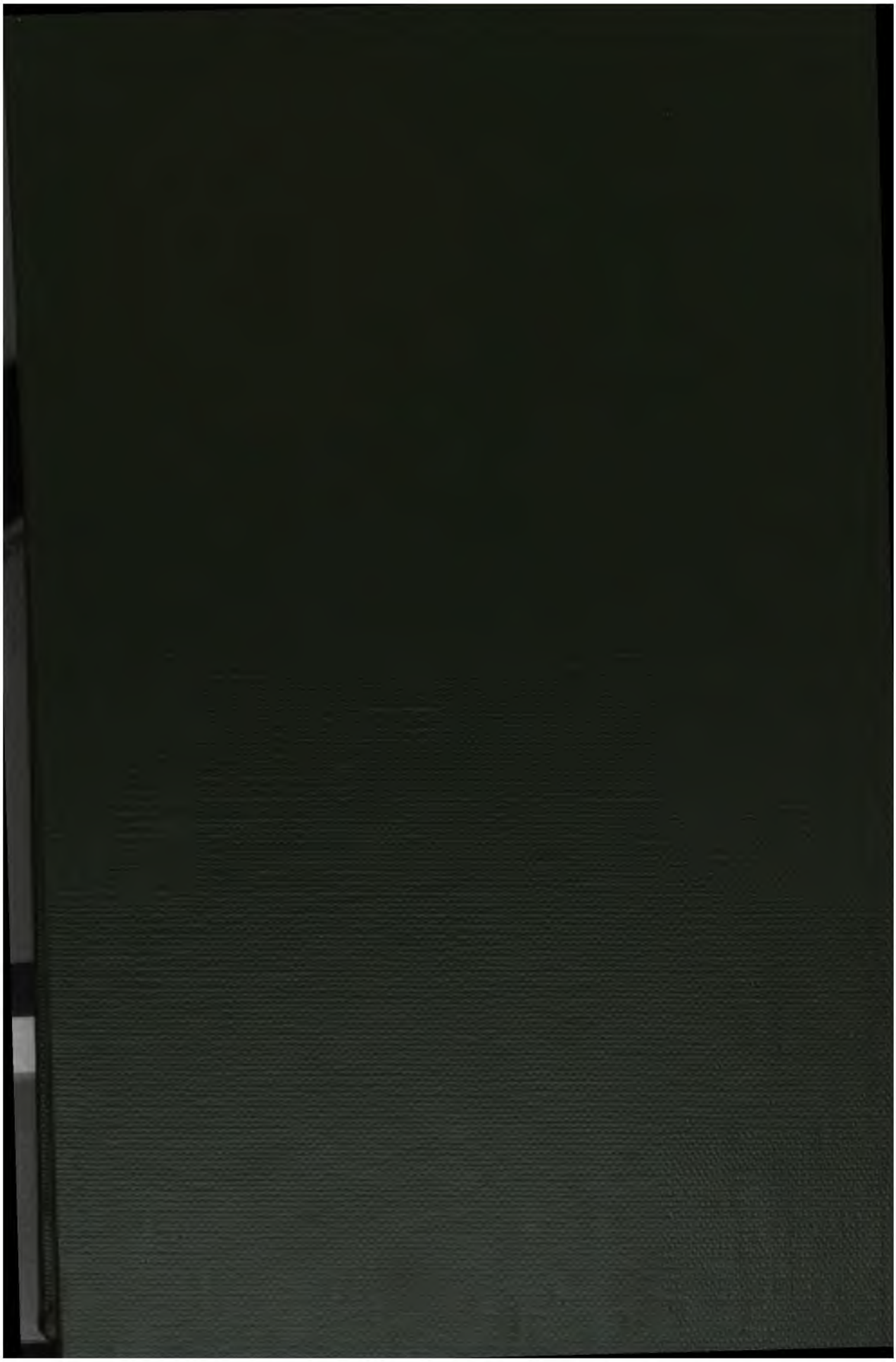
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